The development of a fluorescence for theophylline using the fluoresence capillary fill device (FCFD)

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THE DEVELOPMENT OF A FLUOROIMMUNOASSAY FOR THEOPHYLLINE USING THE FLUORESCENCE CAPILLARY FILL DEVICE (FCFD)

Submitted by

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for the degree of PhD

of the University of Bath

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The research work contained within this thesis has been carried out in the School of Pharmacy and Pharmacology, under the supervision of Dr Michael D. Threadgill and Dr Colin W. Pouton

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ABSTRACT

A homogeneous one step immunoassay for theophylline using the FCFD, a novel optical immunosensor has been described. The assay relies on the phenomenon of an evanescent wave which has the ability of distinguishing bound species from those in free solution without a prior separation step.

The FCFD based assay for theophylline involved competition between a fluorescent derivative of theophylline and theophylline in sample for a limited number of antibody binding sites. The theophylline assay, therefore, required fluorescent analogues of theophylline for use in the FCFD. Synthetic approaches to such analogues involved reaction of alkane-α,ω-diamines with 6-chloro-1,3-dimethylpyrimidine-2,4-dione. Nitrosation of the corresponding 6-(ω-Cbzaminoalkylamino)pyrimidines and subsequent thermal cyclisation afforded the 8-(Cbzaminonoralkyl)theophyllines in good yields. These were deprotected and the intermediate amines were coupled with fluorescein-5-isothiocyanate under conditions of controlled pH to give the required fluorescent derivatives.

Thus, in assessing the full performance of the assay, specific binding isotherms were constructed in both Tris buffer and serum albumin together with the kinetics of binding. A standard curve for theophylline was thus constructed using the analogue having the highest affinity for the anti-theophylline antibody and covered the clinically relevant region of theophylline (0-50 $\mu g\ ml^{-1}$) without interference from theophylline metabolites and structurally related dietary xanthines. The potential for routine use of the FCFD device in clinical assay of theophylline has been demonstrated.

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ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Cyclic Adenosine Monophoshate
cGMP	Cyclic Guanosine Monophosphate
CNS	Central Nervous System
DCC	Dicyclocarbodiimide
DMF	N,N'-dimethylformamide
DMS	Dimethyl sulphate
DTAF	Dichlorotriazinylfluorecein
EIA	Enzyme immumoassay
FCFD	Fluorescence Capillary Fill Device
FIA	Fluoroimmunoassay
FITC	Fluorescein isothiocyanate
GC	Gas Chromatography
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
I.R.	Infra-Red
J	Coupling constant
K_a	Affinity constant
M.S.	Mass Spectra
m/z	mass to charge ratio
NMR	Nuclear Magnetic Resonance
NSB	Non-Specific Binding
PBSTA	Phosphate-buffered saline/Tween/Azide
PDE	Phosphodiesterase
PGE	Platelet Growth Ehancer
PGF	Platelet Growth Factor
Ph	Phenyl radical C_6H_5-

pK_a	measure of acid strength
R	alkyl
SPR	Surface Plasmon Resonance
Tris	Tris(hydroxymethyl)aminomethane
Tween	Polyoxyethylenesorbitan
U.V.	Ultra violet

To My Parents

CHAPTER 1

THE BIOLOGY OF THEOPHYLLINE

1.1 Introduction

The detection of low molecular weight analytes, particularly drugs and biological peptides at concentrations found in blood is of great importance in drug therapy with clinical doses of such materials appearing at much lower concentrations than their large molecular counterparts (*eg.* DNA, proteins, bacteria and viruses). The use of analytical systems to measure such concentrations is of great importance to clinicians where rapid assay systems provide great diagnostic and therapeutic benefit to both patients and practitioners. One such drug of considerable clinical importance is theophylline, a low molecular weight analyte (Mr 180.1), which requires constant monitoring due to its narrow therapeutic window.

Theophylline is a naturally occurring alkaloid which is primarily found in tea, coffee, cola beverages and chocolate. It has a close structural relationship to caffeine and theobromine (Figure 1) and belongs to a class of compounds known as the methylxanthines.

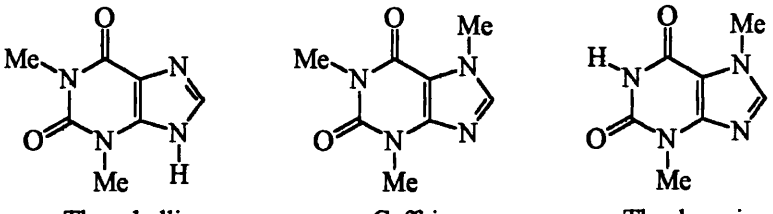
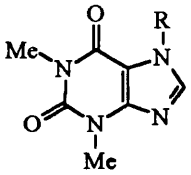


Figure 1. Theophylline and related methylxanthines

The methylxanthines share several pharmacological actions in common, including stimulation of the central nervous system (CNS) (Rall 1985), relaxation of smooth muscle (Karlsson and Persson 1981), inhibition of phosphodiesterase (Kuehl *et al.*, 1987), antagonism of adenosine activity (Sattin and Rall, 1970) and diuresis (Piafsky and Ogilvie, 1975). These effects are summarised in Table 1.

Table 1.- Biological activities of 7-alkyl-1,3-dimethylxanthines



Generic name	N-7 Functional group (R)	Use	In-vitro potency
Caffeine	$-CH_3$	CNS stimulant	0.2
Theophylline	-H	Broncholytic	1.0
Diprophylline	$-CH_2(OH)CH_2OH$	Broncholytic	0.1
Etophylline	$-CH_2CH_2OH$	Cardiotonic	0.14
Proxyphylline	$-CH_2CH(OH)CH_3$	Cardiotonic/ Broncholytic	0.15

The various stable derivatives of theophylline are formed by substitution of larger functional groups (R) on the 7-nitrogen of the 1,3-dimethylxanthine structure. *In vitro* potency was determined from the ability of these compounds to relax human tracheal strips (Svedmyr 1977). Table adapted from Hendeles and Weinberger (1983).

Theophylline, however, is the principal drug of choice in the treatment of *status asthmaticus.* It has been recognised as a potent bronchodilator for the relief of acute asthmatic symptoms, chronic obstructive airways disease and related lung disorders (Hendeles and Weinberger, 1983, Rall, 1985). Subsequently, the use of theophylline has come to be regarded as the first-line therapy for acute asthmatic attacks and as a prophylactic agent in controlling the symptoms of chronic asthma (Weinberger *et al.*, 1981). Theophylline has also been indicated as a cardiac muscle stimulant and has been used to reduce the frequency of occurrence of neonatal apnoea (Rall, 1985) (Table 1).

The physiological effects of theophylline have been correlated with its circulating plasma concentration (Hendeles and Weinberger, 1982), which is not only dependent on its half life but varies considerably between individuals (Jenne *et al.*, 1977, Weinberger *et al.*, 1981, Rowe *et al.*, 1988). Therefore, for adequate control of adult asthma, the therapeutic concentration range of theophylline has been optimised at 10-20 $\mu g\ ml^{-1}$ (Weinberger *et al.*, 1981, Hendeles and Weinberger, 1983, Self and Abou-Dhala, 1994). For neonatal apnoea, therapeutically useful steady states are achieved at lower concentrations with the therapeutic range quoted at 5-10 $\mu g\ ml^{-1}$ (Weinberger *et al.*, 1981).

The association between the peak serum concentration of theophylline and its toxicity has been established by numerous studies (Jacobs *et al.*, 1976, Helliwell and Berry, 1979, Hendeles and Weinberger, 1982, Bertino and Walker, 1987). Theophylline toxicity is seen to occur in patients primarily receiving long-term therapy who develop toxic effects either acutely or chronically as a result of decreased drug clearance or inappropriate drug usage (Bertino and Walker, 1987).

The most common side effects of theophylline administration are mild caffeine like symptoms including anorexia, nausea, vomiting and mild CNS stimulation (Rall, 1985) and occur at concentrations above 20 μg ml^{-1}. Fatal intoxication occurs at serum concentrations above 35 μg ml^{-1} and include seizures, brain damage and death in some instances (Hendeles and Weinberger, 1983), particularly with intravenous therapy.

Therefore, in view of the large inter-patient variability in the elimination of theophylline, effective therapy is best achieved by monitoring both the response of the patient and the concentration of theophylline in the plasma (Rowe *et al.*, 1988). Measurement of theophylline concentration in serum is essential for optimal management of asthma, both chronic and acute, and provides a means of assessing dosage and diagnosing toxicity (Jacobs *et al.*, 1976).

Of the various analytical methods available, high pressure liquid chromatography (HPLC) and immunoassay techniques offer the greatest advantage. They are rapid, specific and require small sample volumes. The enzyme immunoassay technique (EMIT) has gained widespread popularity over time since it is more rapid than conventional techniques and is adaptable for large batches of samples.

Currently, the use of immunosensors for monitoring concentrations of theophylline in plasma has gained much momentum with the trend towards a one step assay system that is not only quick, easy to use and highly specific but conforms to patient compliance. The ultimate aim is, therefore, to develop a testing device that is feasible and accurate, and rapid to use.

1.2 The physical chemistry of theophylline

Theophylline (1,3-dimethylxanthine) was first isolated from tea leaves by Kossel in 1888 as a monohydrate salt with m.p. 272-274°C. A natural CNS stimulant and smooth muscle relaxant, theophylline has been prescribed as an effective bronchodilator since 1937 when Hermann and, subsequently, Greene showed the appreciable effect of intravenous theophylline in alleviating the symptoms of acute asthma (Greene *et al.*, 1937).

Presently the use of theophylline has been restricted, not only by its dose limiting toxicity but by its poor solubility in water (Martindale, 1989). This lack of solubility is attributed to the relatively hydrophobic nature of theophylline which is related to its chemical structure and governed by two physiochemical factors. Firstly, theophylline forms only weak intermolecular hydrogen bridges with water, in part due to the presence of the two N-Me groups at positions 1 and 3, which cannot hydrogen bond, with one free acidic proton at position 9 (pK_a 8.6 - refer to Table 1.1) which forms weak hydrogen bonds and enhances the hydration of the theophylline moiety (Yanuka *et al.*, 1986).

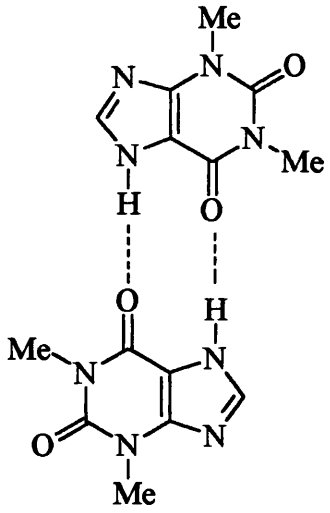


Figure 1.1. The H-bonded dimerisation of theophylline

The second effect is due to base stacking which is thought to contribute to the insolubility of most xanthines and, stabilises the structure of theophylline in the crystalline state (Bruns and Fergus, 1989) causing dimerisation (Figure 1.1). The solubility of theophylline is therefore 1 part in 120 at room temperature but it is freely soluble in hot water (British Pharmacopoeia, 1994).

Table 1.1. The pK_as of theophylline and analogues

	pK_a (acid)	pK_a (base)
Caffeine	—	0.6
Theophylline	8.6	0.3
Theobromine	10.05	0.12

Subsequently, the aqueous solubility of theophylline has been enhanced by complexation with a variety of compounds, in particular with the metal salts of organic acids. These include such salts as calcium salicylate, sodium glycinate and sodium acetate. These are not only commercially available as salt complexes of theophylline but also have medical use as diuretics and cardiac stimulants. However, the derivatives of theophylline which have the greatest therapeutic importance are those containing one or two molecules of an alkylamine. The most important of these is aminophylline which is prepared from ethane-1,2-diamine (Figure 1.2) and contains the equivalent of 85% anhydrous theophylline (Hendeles and Weinberger, 1981).

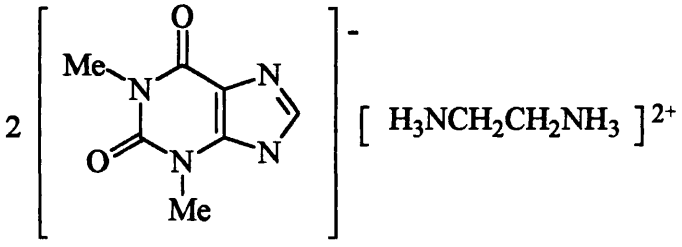
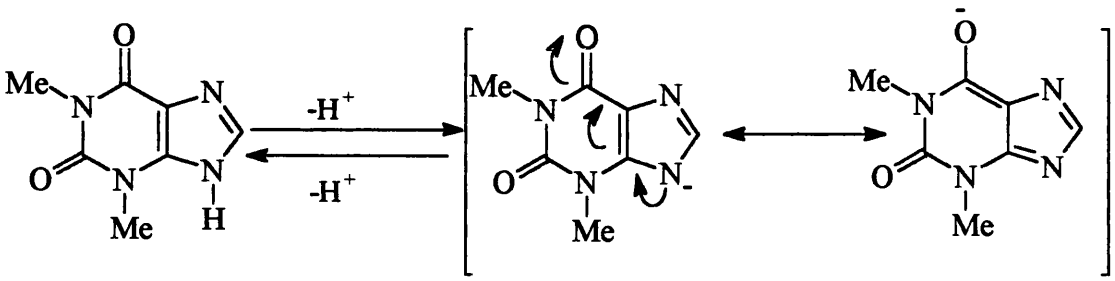


Figure 1.2. The structure of aminophylline

The formation of such complexes is achieved at high pH and is the result of the tautomeric shift in the 9-NH proton (Scheme 1) of theophylline which, as an imidazole derivative, possesses a weak acidity (Table 1.1) and is easily forms salts with bases.



Scheme 1. The tautomeric structure of theophylline and its conjugate base

However, at physiological pH, these theophylline complexes dissociate rapidly and are no more than mixtures of the parent theophylline and the bases to which complexation occurred (Weinberger *et al.*, 1981, Rall, 1985).

1.3 Pharmacological actions of theophylline

The use of theophylline in the treatment of acute bronchial asthma and obstructive airways disease has been available to clinicians for almost a century. However, despite its widespread use as an effective bronchodilator and CNS stimulant, theophylline is far from ideal in that it suffers from a relatively low therapeutic index (Buckle *et al.*,1994). Headache, nausea and vomiting are common dose-limiting side effects with excessive dosing leading to cardiac arrhythmias, convulsions, seizures and in some instances death.

Controversy still exists concerning the molecular mechanisms by which theophylline exerts its therapeutic effect. However, inhibition of cyclic nucleotide phosphodiesterases, resulting in an increase of concentrations of intracellular cyclic adenosine monophosphate (cAMP) (Miech *et al.*, 1979, Torphy and Undem, 1991) which leads to bronchodilation, is one of the mechanisms favoured. Phosphodiesterases (PDEs) act by hydrolysing cyclic AMP and cyclic GMP (cGMP), which are generated from ATP and GTP by the action of adenylate cyclase and guanylate cyclase, to their non-cyclic monophosphates. Therefore, inhibition of phophodiesterase activity increases the concentrations of cyclic nucleotides in the cytoplasm, resulting in relaxation of bronchial smooth muscle and subsequent bronchodilation (Figure 1.3).

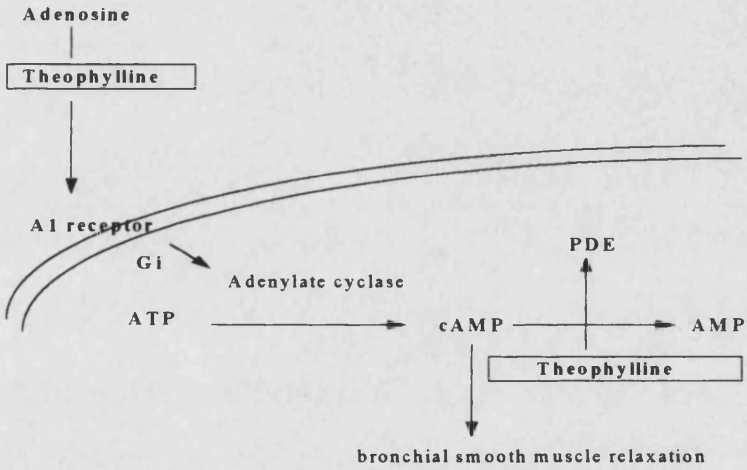


Figure 1.3. The pharmacological action of theophylline

However, theophylline appears to cause smooth muscle contraction at 10- to 20-fold lower concentrations than required for PDE inhibition *in vivo* (Miles and Weinberger, 1983, Svedmyr, 1988, Martindale, 1989), and, therefore appears to be a relatively weak inhibitor of PDE isoenzymes (Daly *et al.*, 1981, Rall, 1982, Rall, 1985, Buckle *et al.*, 1994).

In addition, it has been shown that PDE inhibitors such as dipyridamole, although possessing a greater potency, have no bronchodilatory activity. Findings by Heaslip (1994) suggest that the action of multiple PDE isoenzymes (PDE I-V) (Nicholson *et al.*, 1991), which are not only differentially distributed in cells but possess a degree of selectivity for cAMP or for cGMP, are capable of hydrolysing both cyclic nucleotides (Torphy and Undem, 1993).

In respiratory smooth muscle, functionally relevant cAMP metabolism occurs principally by two distinct PDE isoenzymes (PDE-III and PDE-IV). Recent observations by Heaslip (1994) that PDE-IV is an important regulator of cAMP metabolism in both inflammatory cells and respiratory muscle have resulted in renewed interest in the therapeutic potential of PDE inhibitors as anti-asthmatic drugs.

Theophylline has also been shown to induce bronchodilation, *via* activation of adenylate cyclase, through blockade of adenosine receptors (Sattin and Rall, 1970, Hopwood *et al.*, 1985) at concentrations within the range for therapy of asthma (Fredholm and Persson, 1982). Adenosine (Figure 1.4) is a naturally occurring purine nucleoside that is released in the airways after provocation by allergens (Svedmyr, 1988) and has been implicated in events related to both inflammation and immune response (Jacobson *et al*, 1992).

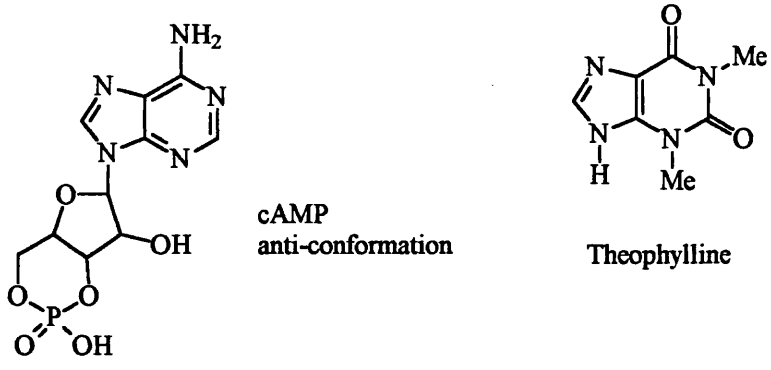


Figure 1.4. The structures of adenosine and theophylline

Although adenosine can affect a variety of physiological functions including the cardiovascular, gastrointestinal and nervous systems (Daly *et al.*, 1981, Bruns and Fergus, 1989), particular attention has been directed over the years towards actions which might lead to clinical applications (Daly, 1982).

The antispasmodic and vasodilatory actions of adenosine appear to be linked to smooth muscle depressant effects which involve activation of two types of membrane receptor, A_1 and A_2 with inhibitory and stimulatory actions on the adenylate cyclase system. The A_1 receptor has been linked to an inhibitory G protein (G_i) that reduces the levels of adenylate cyclase, while the A_2 receptor is associated with a stimulatory protein (G_s) that increases activities of adenylate cyclase. Binding to both receptors is antagonised by theophylline, a weak non-selective adenosine antagonist, which has been shown to have a higher affinity for the A_1 receptor (K_i = 8.5 μM), responsible for mediating smooth muscle contraction, than A_2 receptor (K_i =25 μM). Therefore, the bronchoconstrictor action of adenosine in asthmatic patients and its antagonism by theophylline, at concentrations lower than required for bronchodilation, represents an important area of research (Daly, 1982).

Other mechanisms which have gained much favour in establishing the bronchodilatory activity of theophylline include antagonism of the effect of protaglandins PGE_2 and $PGF_{2\alpha}$ on smooth muscle *in vitro* (Horrobin *et al.*, 1977), stimulation of catecholamine release, the mobilization of intracellular calcium and supression of inflammatory activity through elevation of cAMP (Torphy and Undem, 1991, Teixeira *et al.*, 1994).

Subsequently, seven effects of theophylline have been proposed to be of therapeutic importance in asthma:

(1) Bronchial smooth muscle relaxation;
(2) Increase of mucociliary transport;
(3) Inhibition of the release of mediaters;
(4) Supression of permeability oedema;
(5) Decrease of pulmonary hypertension and increase in right ventricular ejection fraction;
(6) Improved contractility of fatigued diaphragmatic muscle;
(7) Central stimulation of ventilation.

The most important effect is bronchial smooth muscle relaxation, but the other effects may contribute to the antiasthmatic effect especially during maintenance therapy and as a prophylactic agent (Svedmyr, 1988).

1.4 Pharmacokinetics

1.4.1 Absorption and distribution

Theophylline is rapidly and completely absorbed in the gastrointestinal tract, particularly as a disintegrating tablet. However, theophylline is poorly absorbed slow release tablets and suppositories with rectal solutions having the best bioavailability. Following oral administration theophylline is rapidly and completely absorbed with peak plasma concentrations being attained within 2-3 h (Hendeles and Weinberger, 1982).

At steady state plasma concentrations the drug is distributed rapidly into peripheral tissues with of a volume of distribution of 05 L kg^{-1}. This volume is seen to be higher in neonates and diseased states, a phenomenon which is associated with a low plasma albumin concentration (Piafsky *et al.*, 1976). In normal individuals theophylline is about 40% bound to serum albumin (Buss *et al.*, 1983) however, this figure has been quoted as high as 60% by some studies (Vallner *et al.*, 1979, Hendeles and Weinberger, 1982). It was also apparent from studies performed by Shaw *et al.*, (1982) that serum binding of theophylline was influenced by a number of factors including changes in pH (Vallner *et al.*, 1979), temperature and the nature of the buffer solution.

1.4.2 Metabolism and Elimination

Theophylline is eliminated by hepatic biotransformation *via* two distinct and parallel pathways into mostly inactive metabolites that are rapidly excreted in the urine. Approximately 10% of the drug remains unchanged with 85-90% of the dose of theophylline being metabolised by the cytochrome P450 enzyme system (Campbell *et al.*, 1987, Taburet and Schimdt, 1994).

This metabolism involves both first order and capacity limited pharmacokinetic processes, with the major metabolic routes involving both the 8-hydroxylation of theophylline to 1,3-dimethyluric acid, which represents 45-55% of the total clearance of the drug (Tang-Lui *et al.*, 1980, Jonkman and Upton, 1984) and 1-methyluric acid representing 20-25% of the dose (Figure 1.5).

The other metabolite through the demethylation pathway is 3-methylxanthine which represents 13-16% of the total dose of absorbed theophylline.

Conversion of theophylline to caffeine is observed in neonates and children and also in adults with hepatic failure (Dan-Shya *et al.*, 1981).

It has been observed by Wilkinson and Shand (1975) that the hepatic clearance and terminal elimination half-life of theophylline are independent of changes in hepatic blood flow but are highly sensitive to the capacity of the liver to metabolise the drug. Therefore, factors that influence the microsomal oxidative enzymes, demethylation, hepatic uptake or the enzyme xanthine oxidase influence elimination of theophylline (Jonkman and Upton, 1984). Also, variability in clearance appears to be primarily due to physiological abnormalities and differential rates of heparic biotransformation which changes with age, smoking, genetic factors, diet and health (Svedmyr, 1988), Table 1.2.

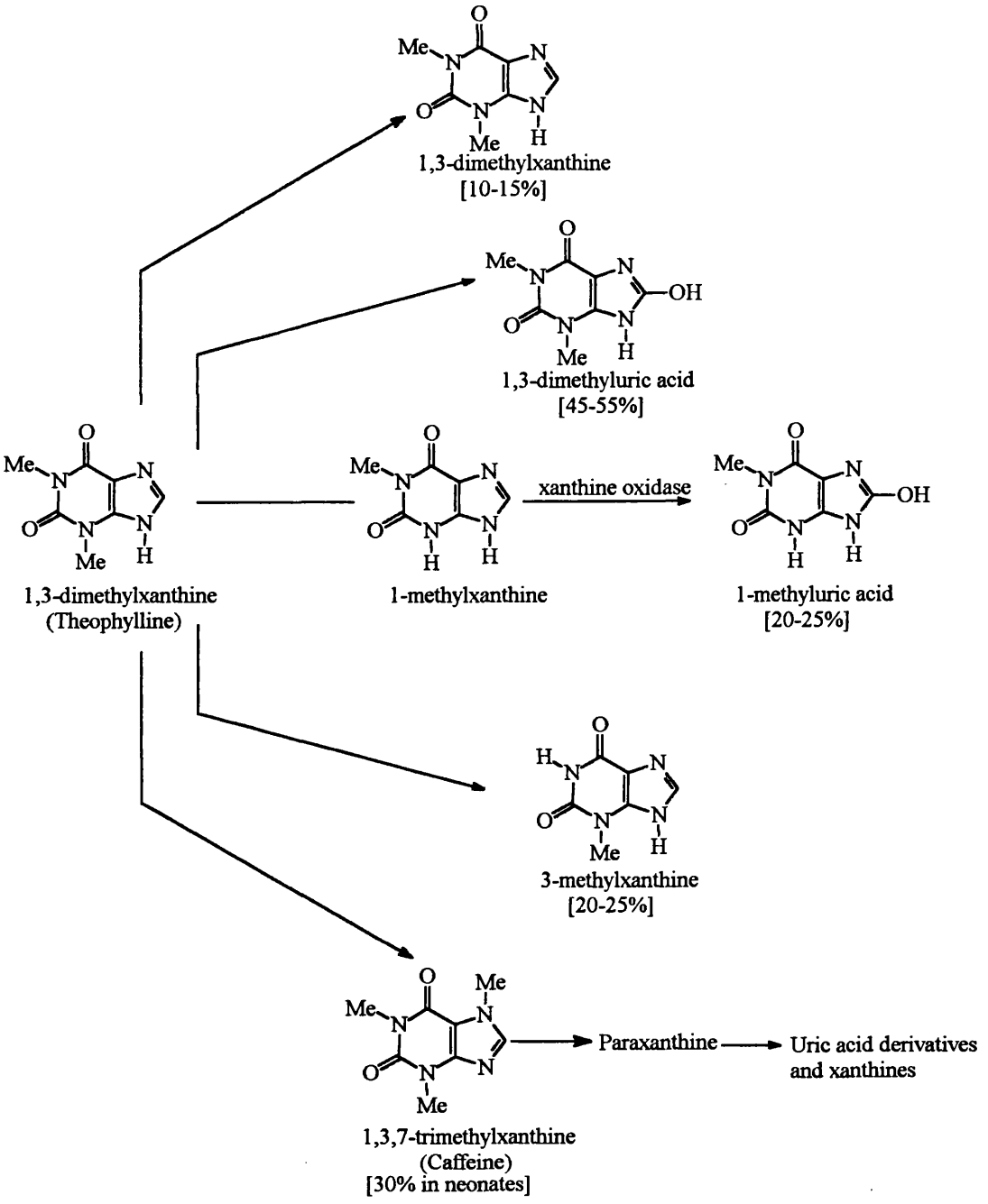


Figure 1.5. The metabolism of theophylline

Table 1.2. Non-genetic factors associated with theophylline clearance

Increased clearance	Decreased clearance
Enzyme-inducing drugs	Enzyme-inhibiting drugs
phenobarbitone	cimetidine
rifampicin	oral contraceptives
phenytoin	erythromycin
Smoking	Methylxanthines
Marihuana	Age
Barbecued meat	Antiviral vaccines
High protein, low carbohydrate diet	Viral infections
Medical coal	Liver cirrhosis
Youth	Congestive heart failure
	Respiratory insufficiency
	Fever

However the single most important variable factor, is genetic, which accounts for a 6-fold variation in clearance in the population (Svedmyr, 1988). Therefore, in order to administer theophylline as an effective bronchodilator, doses must first be individualised.

1.5 Efficacy

The bronchodilator effect of theophylline is proportional to the log of the plasma concentration over a range of 5-20 $\mu g\ ml^{-1}$ with optimal plasma concentration being aimed at 10-20 $\mu g\ ml^{-1}$ for adult asthma. For effective control of neonatal apnoea plasma theophylline concentrations are maintained at 5-10 $\mu g\ ml^{-1}$ (Hendeles and Weinberger, 1981). However, although there is great inter-patient variability and complex pharmacokinetics, theophyline is not only an effective bronchodilator for acute asthmatic conditions but also a prophylactic agent in the management of asthma at optimal concentrations. Although theophylline is an effective bronchoplasmic agent at such concentrations, its therapeutic margin is very low and related to its dose limiting side effects including nausea, vomiting, cardiac arrhythmias, seizures and death. Therefore, in order to achieve the maximum benefit from theophylline for chronic asthma treatment, serum concentrations should be maintained within the therapeutic range around the clock.

Fluctuations in serum concentrations are a function of the rate of absorption of the product, the rate of elimination of the drug from the patient, and the dosing interval selected (Hendeles and Weinberger, 1983). When theophylline is used as an effective bronchodilator, the goal is to obtain a therapeutic serum concentration rapidly so that symptoms are relieved as quickly as possible.

This goal is best accomplished with an intravenous loading dose, although rapidly absorbed formulations such as oral solutions, plain uncoated tablets or rectal solutions may be adequate for patients at home or for those hospitalised with less severe symptoms.

1.5.1 The toxic effects of theophylline

Theophylline has been the drug of choice for the treatment of asthma in the United States since 1937. However due its narrow therapeutic window (10-20 μg ml^{-1} in adults) and the resulting toxicity associated with plasma concentrations greater than 20 μg ml^{-1}, its use is severely limited (Table 1.3). Various drug toxicities have been described including nausea, cardiac arrhythmias and potentially lethal seizures. Generally symptoms of theophylline toxicity affect the cardiac, gastrointestinal or neurological systems. with the severity of the toxic reaction having been related to the patients peak serum concentration, as outlined in Table 1.3 (Hendeles and Weinberger, 1983).

Table 1.3. The efficacy and toxicity of theophylline dosage

Concentration mg l^{-1}	Efficacy	Toxicity
5	Minimal	
10	"	
20	Optimal	Gastrointestinal upset
40	"	Nervousness
60	"	Arrhythmia
	"	Convulsions

Jacobs *et al.* (1976), in assessing the relationship between dosage, serum concentration and toxicity, observed clear-cut toxic reactions relating to serum concentrations between the range 15.8-49.5 μg ml^{-1}.

Mild toxic conditions were observed at serum concentrations below 25 μg ml^{-1}, in particular relating to nausea and vomiting. Serious toxicity has been observed at serum levels greater than 35 μg ml^{-1} resulting in such conditions as tachycardia, cardiac arrhythmias, electrolyte abnormalities including hyperglycemia and hypercalcemia, seizures and death.

However, Hendeles and Weinberger (1982) have observed focal and generalised seizures at plasma concentrations as low as 25 μg ml^{-1} without prior signs of toxicity while Zwillich *et al.* (1975) reported serum concentrations of 54 μg ml^{-1} among patients with seizures, compared with 35 μg ml^{-1} for patients with minor adverse effects.

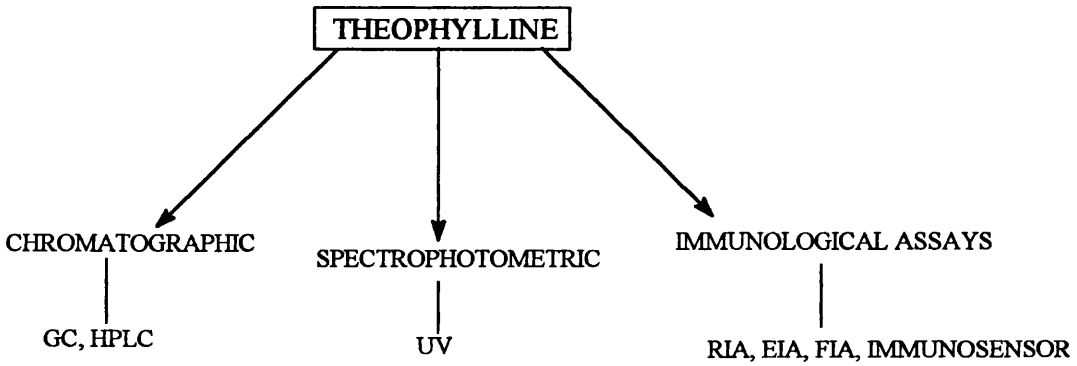
Cardiac toxicity has been most evident with adults, while tachycardia occurred in premature newborns at concentrations above 10 μg ml^{-1}. Hypotension and sudden cardiac arrest has been associated with rapid intravenous dose of theophylline while among infants and children fatal toxicity has been as a result of therapeutic misadventure, particularly when multiple adult doses were administered (Hendeles and Weinberger, 1983). Further findings by Shannon (1994) reveal a greater risk of toxicity in elderly patients, which have been attributed to altered elimination rates as a result of concomitant heart and liver disease and through increase in drug reactions.

It must be stated that minor symptoms of toxicity such as nausea and vomiting cannot be relied upon as a dosing end-point (Hendeles and Weinberger, 1983). Only serum theophylline measurements provide a means of forewarning the clinician of impending life-threatning toxicity.

1.6 Monitoring of theophylline serum concentration

The association between serum theophylline concentrations and efficacy, as well as toxicity, has been clearly defined by numerous studies (section 1.4) with a safe upper limit of 20 μg ml^{-1} and a much softer lower limit between 3-10 μg ml^{-1} (Hendeles and Weinberger, 1983). Adequate control is generally quoted between 10-20 μg ml^{-1} but in cases of toxicity, levels as high as 60 μg ml^{-1} may be encountered. Due to this narrow therapeutic range and a wide variation in inter-patient half life, related both to the drugs clearance and hepatic absorption, monitoring of serum theophylline levels is considered necessary for safe and effective use of the drug.

Various methods are available for the measurement of plasma theophylline concentrations including spectrophotometry, chromatography (GC and HPLC) and immunoassays involving radiolabels (RIA), enzymes (EIA) and fluorophores (FIA) as shown in scheme 1.1.



Scheme 1.1. The analytical methods available for the measurement of serum theophylline levels.

The non-immunological methods, although useful, all have disadvantages primarily owing the requirement of large sample volumes, lack of selectivity and the use of technically complex and time-consuming sample preparations.

Although being more labour-intensive HPLC does offer the advantage of measuring other metabolites including the closely related methylxanthine, caffeine (Rowe *et al.*, 1988).

On the other hand immunoassays are simple, permit direct analysis with small sample volumes (25 µl in EIAs), specific, have high sensitivity (particularly in the use of heterogeneous systems) and can be automated to deal with large batch of samples. The various published methods for monitoring theophylline serum levels are outlined in Table 1.4 and show a growing trend in the use of homogeneous immunoassay systems for the monitoring of clinically important drugs such as theophylline.

Table 1.4. Methods for monitoring theophylline concentrations

Assay	Detection method	Label used	References
Fluorescence immunoassay (FIA)	Fluorescence emission	Fluoresceinthiocarbamyl-ethylenediamine	Hodgkinson *et al.*, 1985
Fluorescence polarisation (FPIA)	Change in fluorescence polarisation	Umbelliferone	Li *et al.*, 1981a
Enzyme multiplied immunoassay (EMIT©)	Spectrophometric	glucose-6-phosphate	Chang *et al.*, 1982
Apoenzyme reactivation immunoassay (ARIS©)	Spectrophometric	FAD	Rupchock *et al.*, 1985 Lindberg *et al.*, 1985
Ligand displacement immunoassay	Spectrophometric	β-galactosidase	Hinds *et al.*, 1984
Immunosensor (Flowinjection)	Electrochemical	Alkaline phosphatase	Palmer *et al.*, 1993
Immunosensor (Amperometric)	Electrochemical	NADH	Athey *et al.*, 1993

The need for continuous monitoring without operator skill as well as rapid data collection has, therefore, led to the development of immunosensor technology. In the next chapter, the use of immunosensors as viable alternatives to conventional homogeneous assay systems is explored.

CHAPTER 2

IMMUNOSENSOR ASSAY FOR THEOPHYLLINE

2.1 Introduction to Immunosensors

Immunoassays offer a highly sensitive and selective approach to the detection and quantification of trace metabolites arising from the interaction between antibody and antigen. The technique is highly specific and has found widespread use in diagnostic medicine in the detection of analytes such as hormones, clinical disease markers, drugs, bacteria and environmental pollutants (Robinson *et al.*, 1987).

Following the pioneering work of Yalow and Berson in the early 1960s, immunoassay procedures have rapidly assumed a major role in the clinical investigation of disease, monitoring therapy and environmental analysis (Leech, 1994). The high specificity which typifies an antigen-antibody reaction has led to extensive research in the area of immunosensing. Subsequently, the demand for immunoassays and the remarkable progress in immunochemical technology (Aizawa, 1991) has led to the integration of current biosensor technology with both immunology and biotechnology.

Immunosensors take advantage of the high selectivity provided by the molecular recognition of antibodies, which, in principle, can be obtained against an unlimited number of determinants. An immunosensor is, therefore, described as a device incorporating a biological molecular recognition component (*e.g.* an antibody or antigen) connected to a transducer (the sensor) which can output an electronic signal that is proportional to the concentration being sensed (Byfield and Abushenka, 1994).

A range of combinations of receptor-transducers have been proposed and listed in Table 2 with detailed reviews of such technological variation widespread in the literature (Cass, 1990, Turner *et al.*, 1991).

Table 2. The variation in biosensor technology

Transduction technology	Biological element
Electrochemical	Enzymes
Amperometric	Enzyme systems
Potentiometric	Membranes
Conductimetric	Cells
Optical	Microorganism
Fluorescence	Organelles
Absorbance	Cofactors
Acoustic	Tissues
Calorimetric	Antibodies
Piezoelectric	Receptors
Mechanical	

The biological component of the biosensor element confers specificity and is divided into two distinct groups (a) catalytic, which includes enzymes, microorganisms and tissues and (b) non-catalytic, comprising of antibodies, receptors and tissues (Griffiths and Hall, 1993). The nature of the interaction of the biological element has a major impact on the current technologies available, in particular those utilising a wide range of optical and electrochemical systems in conjunction with biological sensing (Byfield and Abuknesha, 1994).

Immunosensors, therefore, exploit the high specificity and sensitivity (Figure 2) which typifies an antigen-antibody reaction and can be applied to both sandwich (non-competitive) and competitive assays.

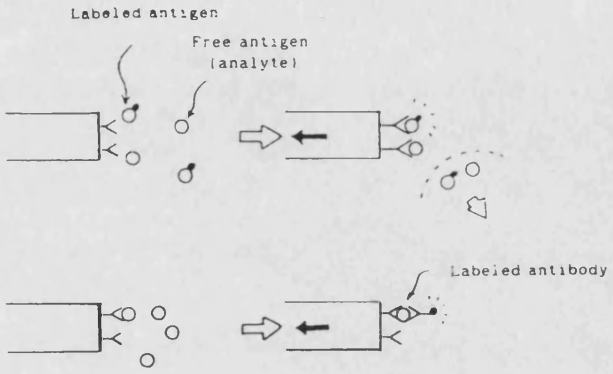


Figure 2. Principle of an optical enzyme immunosensor: (a) competitive and (b) sandwich immunoassays

Both systems are based on the solid phase immobilisation of the biological component (antibody or antigen) on a planar or fibre optic guide and offer a means of detecting antibody binding reactions with sufficient sensitivity and specificity.

Immunosensors can also be utilised for both direct and indirect detection of antibody reactions in immunoassay procedures. Indirect detection, which is by far the most predominant scheme, incorporates a tracer or label which is used to generate a signal (e.g. optical, electrochemical) which enables quantification of the amount of bound antigen relative to unbound. Examples of such tracers are shown in Table 2.1 which include enzymes, fluorescent molecules and electrochemical species.

Table 2.1. Non isotopic immunoassay labels

Category	Label
Chemiluminescent compound	Acridinium ester
	Isoluminol
	Luminol
	Phenathridinium ester
Enzyme	Alkaline phosphatase
	α and β amylase
	Bacterial luciferase
	Catalase
	Firefly luciferase
	β-galactosidase
	Glucose-6-phosphatedehydrogenase
	Glucose oxidase
	Horseradish peroxidase
	Xanthine oxidase
Fluorophore	Coumarin (Umbelliferone)
	Europium chelate
	Fluoram
	Fluorescein
	Lucifer yellow VS
	Phycoerythrin
	Rhodamine B
	Samarium chelate
	Terbium chelate

Subsequently, the incorporation of labels into the antibody-antigen immunocomplex provides a means of achieving highly sensitive immunosensors which can be determined by a variety of methods including amperometric, potentiometric and optical detection (Figure 2.1)

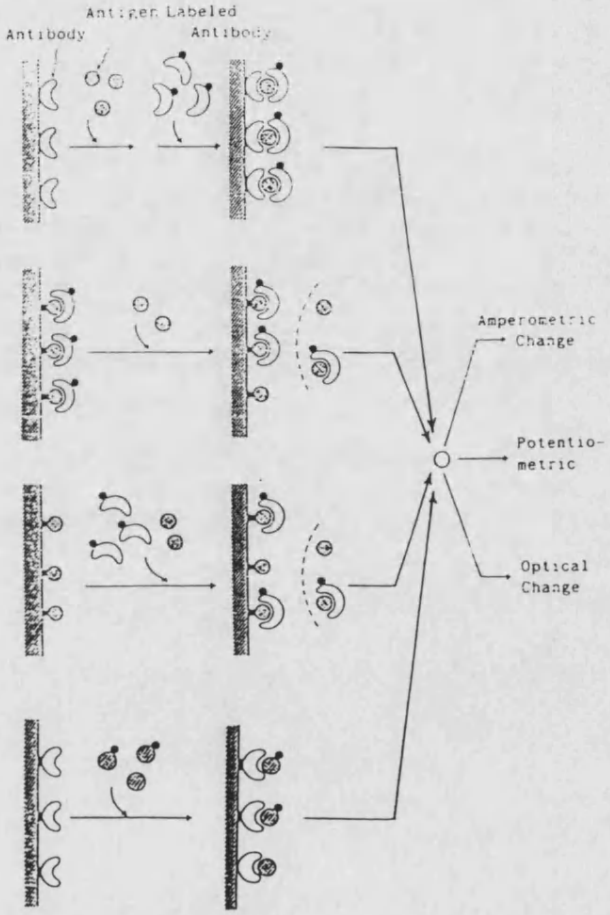


Figure 2.1. Principle of labelled immunosensors

Direct detection methods are not so widespread in immunosensing but offer a means of detecting the immunocomplex directly by measuring the physical changes induced by the formation of the complex (Aizawa, 1991).

The principle is based on the immobilisation of the antibody or antigen to a solid matrix which is sensitive to changes in its surface characteristics to detect immunocomplex formation. Electrode, membrane, piezoelectric material or optically active material may be used to construct the non labelled immunosensor (Figure 2.2) with the immunocomplex formed altering the physical properties of the surface, such as electrode potential, intrinsic piezofrequency or optical properties including formation of evanescent wave and surface plasmon resonance (SPR). Table 2.2 lists the properties of the ideal immunosensor.

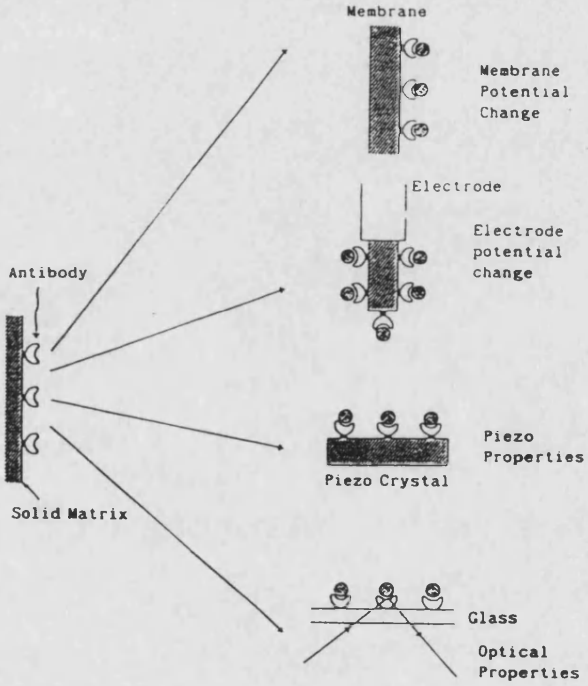


Figure 2.2. Principle of non-labelled immunosensors

Table 2.2. Properties of an ideal immunosensor

(i) Sensitive
(ii) Specific
(iii) Small
(iv) Stable - i.e., long shelf life
(v) Simple to use
(vi) Economical
(vii) Reusable or capable of continuous monitoring
(viii) Insensitive to environmental changes including temp, pH etc.
(ix) Able to sample a defined volume
(x) Homogeneous in use

2.1.1 Heterogeneous systems

The first heterogeneous immunoassays were performed by Yalow and Berson in 1959 using a radioactive label, the radioimmunoassay (RIA). Since its development, the RIA has become one of the most important analytical tools in clinical chemistry and has been applied to monitor a whole host of analytes including hormones, therapeutic drugs (e.g. digoxin and theophylline which require close controls of serum concentration), drugs of abuse (*e.g.* cocaine, morphine) and agents of infectious diseases including AIDS and hepatitis.

The RIA procedure relies on the competition between labelled antigen (Ag*) and unlabelled analyte from serum sample for the Ab (antibody) raised against the analyte in question.

After equilibration the bound fraction is separated from free and the radioactivity present is measured, giving a directly proportional indication of the amount of analyte in sample (Figure 2.3).

Ab + Ag* + Ag → Ab-Ag* + Ab-Ag

from free sample separate and count

Figure 2.3. The principle of RIA

However, although RIA is the most common heterogeneous immunoassay procedure, the recent trend has been towards non-isotopic detection with radiolabels having various self-evident disadvantages, including handling of waste and toxicity, safety and the use of expensive bulky scintillation counters. There is, therefore, a need to design non-isotopic detection methods that are both sensitive and robust (McCapra, 1989).

The most common alternative labels for immunoassays include enzymes (*e.g.* alkaline phophatase, horseradish peroxidase and β-galactosidase), fluorophores (*e.g.* fluorescein and rhodamine) and chemiluminescent probes including luminol and phenanthridium ester.

Enzyme-labelling of antibodies or antigens for amplification of the signal and the detection of antigen levels is the most widely used in non-isotopic procedure. The schemes involved in the most common enzyme immunoassays are the sandwich and competitive type enzyme-linked immunosorbant assay system (ELISA).

However, a number of enzymes have been applied to immunosensing technology, particularly in the assembling of oxygen-sensing electrodes by use of catalase enzyme catalyses the decomposition of hydrogen peroxide into oxygen and water (Ikariyama and Aizawa, 1985).

In heterogenous enzyme immunoassays (EIA), the labelling enzyme activity is measured by amperometry with the oxygen-sensing device and, because only a short time is required for measuring the enzyme label, a rapid and highly sensitive immunoassay can be achieved.

Increased sensitivities may also be achieved using fluorescent labels, such as fluorescein or rhodamine. These have been successfully used in both competitive and non-competitive assays. Most of the commercially available fluorescence-based immunoassays utilise a solid phase antigen or antibody system and involves the separation of the label from endogenous interferences by attachment to a solid surface particle or matrix (Figure 2.4) with current sensitivities are often limited to a range of 10^{-10} M analyte.

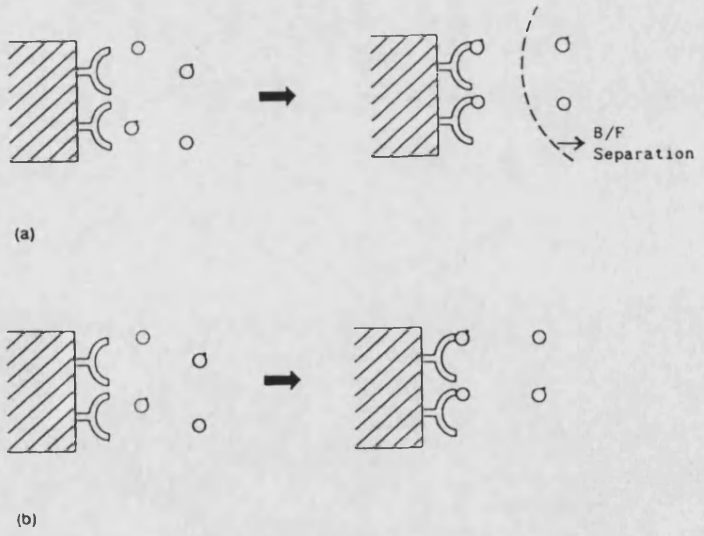


Figure 2.4. Heterogeneous (a) and homogeneous (b) immunoassays

2.1.2. Homogeneous immunoassays

Since the establishment of the radioimmunoassay by Yalow and Berson in 1959 and the subsequent development in heterogeneous immunoassay procedures, several groups have attempted to devise assay systems where a separation step was not required. The first use of a non-separation immunoassay system was performed by Arquilla and Stavitsky in 1956, (Nakamura, 1992a) using erythrocyte-labelled insulin to detect insulin antibodies in diabetes. Subsequently, the first fluorescence-based homogenous immunoassay was developed by Dandliker and Feigen in 1961 (Nakamura, 1992a) for penicillin, using fluorescence polarisation for fluorescein-labelled penicillin bound to antibody

The principle behind many homogeneous assay systems is the competition between the labelled and unlabelled drug for the specific antibody with the resulting antibody-antigen complex having different physical properties to the unlabelled drug. This, therefore, implies a negation for a separation or washing step when the complex is measured in the appropriate analytical system. A comparison between homogeneous and heterogeneous assay systems is outlined in Table 2.3.

Table 2.3. Comparison between Homogeneous and Heterogeneous assay systems

Homogeneous	Heterogeneous
Simple to perform	More complicated assay performance
Easy to automate	Mechanisation difficult
Robust procedure	Higher sensitivities
Limited sensitivity	Wide dynamic range
Limited menu (small antigens)	General applicability
Sensitive to interferences derived from samples (e.g. background)	Less prone to matrix differences

It is apparent from Table 2.3 that the requirement for an easy-to-use, robust and highly sensitive immunoassay system can be applied to both homogeneous and separation assays. However, a review of the literature indicates that, for most commercial assays an easy-to-use robust system requires a non-separation step. Such systems incorporate a variety of labels, of which enzymes (Table 2.4), fluorophores and luminescent compounds are the most common.

Table 2.4. Enzyme labels used in immunoassays

Enzyme	pH optimum	Sp act (U/mg) at 37°C	Mr x 10^3
Alkaline phosphatase	8-10	1,000	100
β-Galactosidase	6-8	600	540
Glucose oxidase	4-7	200	186
Glucose-6-phosphate dehydrogenase	7.8	400	104
Horseradish peroxidase	5-7	4,500	40
Urease	6.5-7.5	10,000	483

The application of enzyme, fluorescent and chemiluminescent labels had led to the development of many homogeneous immunoassays for a range of haptens and protein antigen. These are outlined in Table 2.5.

Table 2.5. Homogeneous assay systems used in clinical chemistry

Basis	Detection of Label	Analyse	Reference
Enzyme Inhibition (EMIT©)	Spectrophotometer	Drugs and proteins	Blake and Gould 1984
(SLFIA) Substrate labelled fluorescent immunoassay	Fluorescence emission	Drugs (theophylline), IgG	Burd 1981
Fluorescence polarisation immunoassay (FPIA)	Polarisation	Penicillin	Dandliker *et al.*, 1973
Fluorescence enhancement	Fluorescence emission	T4	Smith 1977
(PGLIA) Prosthetic group label immunoassay	Spectrophometry	Theophylline	Rupchock *et al.*, 1985
(DELFIA) Delayed enhanced fluorescence immunoassay	Fluorescence emission	Hepatitis B, digoxin	Hemillia, 1993
Chemiluminescence energy transfer	Chemiluminescence	cAMP, IgG	Patel *et al.*, 1983,

2.1.3 Fluorescence immunoassay (FIA)

The use of fluorescent labels to detect immunological reactions has rapidly become standard practice in the clinical investigation of drugs, proteins and hormones (Soini and Hemmila, 1979). The first reported use of a fluorophore as a specific label for biological material was performed by Coons and co-workers (1941) using anthracene isocyanate to label bacterial proteins. Subsequently, the use of fluorescent probes in immunoassay procedures have gained much popularity in clinical chemistry, in effect this is due to the decrease in use of radioisotopes as labels for immunoassay procedures and an increase in the use of non-isotopic labels of comparable sensitivity.

The sensitivity of FIAs are generally quoted in the picomolar range although limitations due to high background fluorescence caused by bilirubin and other compounds in serum, combined with light-scattering and quenching effects, are prevalent in the literature (Byfield and Abuknesha, 1994: Mayer and Neuenhofer, 1994). However, despite these limitations, fluorescent labels are seen as a viable alternative to radioimmunoassays particularly as fluorimetric labelling is relatively inexpensive and non radioactive, being therefore safer, rapid, simple and requiring relatively inexpensive instrumentation. Also, FIAs can be applied to both heterogeneous and homogenous systems particularly in the area of immunosensing *via* use of an evanescent wave (discussed in section 2.2.1). Increased sensitivity of immunochemical reactions is also achieved *via* use of fluorescence polarisation and time resolved fluorescence using lanthanide chelates.

The most commonly used fluorophores in immunoassay reactions are organic ring structures which fluoresce on absorption of light at the excitation wavelength. Fluorescein isothiocyanate (FITC) is the most common fluorescent probe used in labelling proteins. It has a strong fluorescence, a high quantum yield and a fluorescence spectrum with an excitation maximum at 490 nm and emission maximum at 520 nm (Figure 2.5).

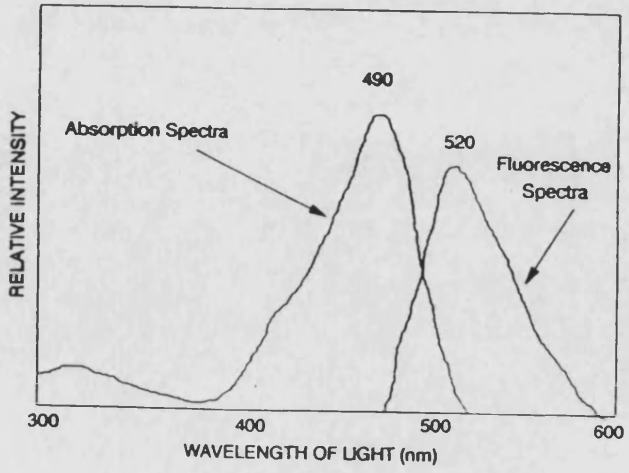
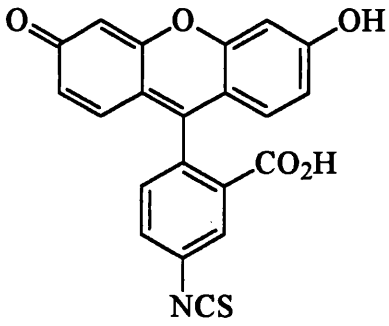
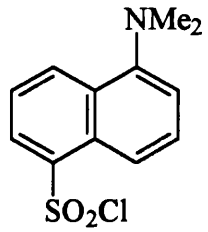


Figure 2.5. Absorption and fluorescence spectra of fluorescein at pH 7.1.

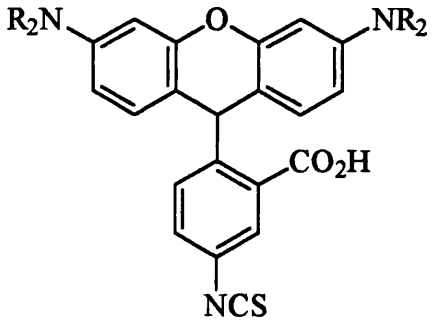
Although FITC has become the fluorophore of choice in most applications (Jolley *et al.*, 1984) its use has been limited, due mainly to the small Stokes shift (20-30 nm) hence scattering causes considerable interference at low concentrations (Nakamura 1992b). Similar properties are also exhibited by rhodamines which belong to the same class of dye, Figure 2.6 shows the structural variation in fluorophores frequently used to derivatise proteins.



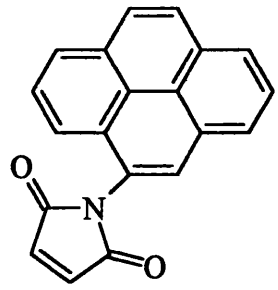
Fluorescein isothiocyanate (FITC)



Dansyl chloride



Rhodamine isothiocyanate B



Pyrene maleimide

Figure 2.6. Structures of common fluorescent labels.

Therefore, for optimisation of a fluorescence-based immunoassay, the probe in question must have;

(a) high fluorescence intensity with a quantum yield approaching unity.

(b) large Stokes shift, ideally >50 nm.

(c) emission wavelength between 500-700 nm so that it can be distinguished from background.

(d) binding to either antibody or antigen must not affect the fluorimetric properties of the probe.

The properties of the most common fluorophores are outlined in Table 2.6.

Table 2.6 showing the properties of selected fluorescent labels

Fluorophore	Quantum yield	Lifetime (ns)	Emission/emission wavelength (nm)	Absorptivity (liters/mol)
Fluorescein isothio-cyanate (FITC)	0.85	4.5	520/420	7×10^4
Rhodamine B isothio-cyanate (R=Me)	0.7	3.0	585/550	1.2×10^4
Dansyl chloride	0.3	14.0	482-520/340	3.4×10^3
Eu-(β-naphthoyl tri-fluoroacetone)$_3$	0.8	500,000	590, 613/340	3×10^4
Phycobiliprotein	0.5-0.98		580-660/550-620	7.0×10^5
Lucifer yellow VS			540/340	
N-(3-Pyrene)-maleimide		100	375, 392/340	3.8×10^4

Fluorescent labels can be utilised in both heterogeneous and homogeneous immunoassay systems.

The procedures involved are particularly useful in both competitive and sandwich type assays where antibody or antigen is immobilised on a solid phase. In heterogeneous assays, the bound fluorescent protein is separated from free fluorescent protein and the resulting fluorescence measured.

In homogenous systems, this washing/separation step is avoided. Examples of such immunoassay procedures include substrate-labelled FIA (SLFIA) (Li *et al.*, 1981b), fluorescence polarisation assay (Li *et al.*, 1981a), DELFIA (Hemmilia *et al.*, 1993) and FIAs by internal reflection spectroscopy for use in optical immunosensing. The technique utilises total internal reflection to excite the fluorescence of FITC-labelled antibody which is bound to hapten-protein conjugate absorbed on a quartz plate in antibody solution (Kronick and Little, 1975). Other homogeneous systems include fibre optic fluorescence immunosensors which utilise the property of an evanescent wave to detect surface bound components (Aizawa, 1991) and the use of a fluorescence capillary fill device (FCFD), a planar optical waveguide (Badley *et al.*, 1987).

2.2 Optical immunosensors

Immunosensing is currently receiving much attention owing its promises of enhanced sensitivity and elimination of costly and tedious separation and incubation procedures (Ahluwalia *et al.*, 1991). Transducing systems coupled to immunosensors for the direct detection of Antibody-Anitigen interactions include potentiometric, piezoelectric, acoustic and optical devices (Lowe, 1989). However, the most recent developments in immunosensing have all been directed towards optical sensing in both direct and indirect systems.

Optical immunosensing techniques have been applied to a wide range of analytes and have been incorporated into planar waveguides and fibre optic immunosensors. Indirect detection of the pregnancy hormone human chorionic gonadotrophin (HCG) was achieved by Christensen *et al.*, (1994) using a planar waveguide utilising a fluorescence immunoassay. The technique has been applied to both competitive and sandwich assays with reported reaction times much shorter than traditional enzyme-based systems. Other optical sensing systems utilising fluorescence-based evanescent wave immunosensors have been reported with the most promising system incorporating a novel capillary fill device (Badley *et al.*, 1987).

However, optical immunosensors for direct sensing have also been developed which also exploit the use of an evanescent wave to probe the interactions within a few nanometres of the sensor surface. These optical sensors are based on devices in which a thin layer (50 nm) of metal is deposited onto the glass surface which, on illumination with a light beam, cause the electrons within the metal surface to resonate. The technique is called surface plasmon resonance (SPR) and is sensitive to changes in the refractive index of the media at the surface.

The technology has already been commercially exploited by the development of the BIA core SPR system by Pharmacia providing a means of attaining real time analysis of biological interactions (Buckle *et al.*, 1993).

2.2.1 Evanescent wave physics

The use of an evanescent wave to monitor antibody-antigen reactions at a quartz liquid interface has gained much popularity in the last ten years, particularly in the design of simple, rapid, homogeneous assay systems.

Several groups (North, 1985, Badley *et al.*, 1987 and Fletcher *et al.*, 1993) have incorporated the unique properties of an evanescent wave into immunosensor design in both direct and indirect systems. However the most sensitive immunosening techniques have been applied to the detection of fluorescent labels in an evanescent field in both planar optical waveguides (Badley *et al.*, 1987, Fletcher *et al.*, 1993, Christesen *et al.*, 1994) and fibre optics.

The generation of an evanescent wave relies on the unique optical characteristics found at the reflecting surface between two transparent media of different refractive indices (Harrick, 1967, Sutherland *et al.*, 1984). Therefore, on travelling within a transparent but optically dense medium (n_1), a light beam is totally internally reflected provided that the incident angle (θ) is greater than the critical angle (θ_c)

$$\theta_c = \text{arc sin } (n_2/n_1)^{-1}$$

The internally reflected light beam generates an electromagnetic 'evanescent wave' close to the reflecting surface in the optically rarer medium (n_2) which is part of the internally reflected light beam (Sutherland *et al.*, 1984). This evanescent wave penetrates a fraction of a wavelength beyond the reflecting surface into the rarer medium (n_2) and decays exponentially with distance from the surface-solution interface.

Therefore, chemical or biological reactions occurring at, or very close, to this interface perturb the evanescent wave, giving rise to a change in signal, which for antibody-antigen reactions can be related to the amount of binding (Byfield and Abuknesha, 1994).

For fluorescence-based systems, biosensing is achieved when there is a change in fluorescence excitation as a result of the interaction by the evanescent wave in this field upon binding of antigen to immobilised antibody (Sutherland *et al.*, 1984) see expression below:

$$E = E_o \exp(-2/d_P)$$

where d_P = the distance required for the electric field amplitude to fall to exp (-1) of its value at the surface (Figure 2.7).

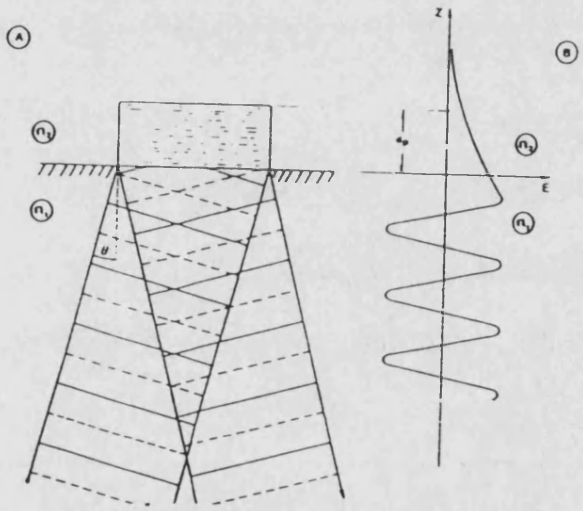


Figure 2.7. Generation of an evanescent wave at an interface between two optical media; (A) where $n_1 > n_2$ and $\theta_1 > \theta_c$, the critical angle at which refraction occurs

Thus, the generation of an evanescent wave allows optical detection of a reaction or of reaction products at the interface between the two media without interference from the bulk solution (Sutherland *et al.*, 1984). The evanescent wave phenomenon allows construction of an active immunosensing transducer which will measure immunochemical reactions on the surface of both optical fibres (Bluestein *et al.*, 1990) and planar waveguides (Parry *et al.*, 1990). Subsequently, unbound molecules in solution beyond this evanescent region are not detected, thus eliminating the need for extensive washing prior to measurement is eliminated.

2.2.2 Surface plasmon resonance

The phenomenon of surface plasmon resonance has been used in immunosensor technology by several groups, since the early part of the 1980s (Nylander *et al.*, 1982, Liedberg *et al.*, 1983, Flanagan and Pantell, 1984), particularly in binding assays. The technique is both simple and direct and offers a means of measuring the antibody-antigen reaction without the need for a separation or wash-out step. Surface plasmon resonance (SPR) like total internal reflection spectroscopy is a technique based on direct sensing of immunological reactions by excitation of surface bound antibodies.

SPR is defined as a collective motion of electrons in the surface of a metal (usually gold) conductor which is excited by the impact of light of appropriate wavelength at a particular angle. Thus resonance only occurs if the momentum vector of the plasmon in the metal surface is equal to the longitudinal component of the momentum vector of the incident light. Therefore to achieve this plasmon excitation the grating surface is illuminated with white light at a fixed angle of incidence θ with the signal generated from this SPR wave detected by measuring the intensity of light reflected off the metal surface (Figure 2.8).

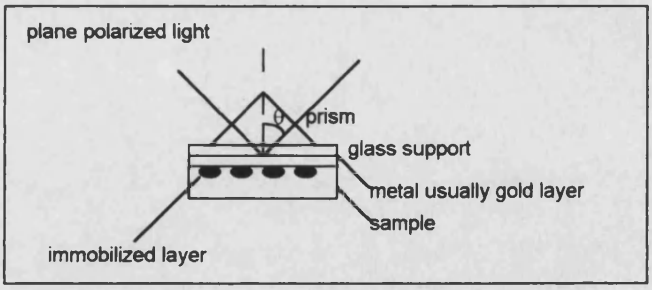


Figure 2.8.

The plasmon resonance, a direct result of the oscillation of electrons in the surface of a metal conductor, results in a high electric field strength close to the metal surface which decays exponentially away from the metal such that it penetrates a short distance into the solution.

Therefore, only changes in refractive index occurring within this field affect the SPR signal and surface plasmons are thus unaffected by properties of the bulk solution and are modulated by changes in refractive index close to the metal surface.

In recent years the use of SPR has become an important feature in biosensing particularly for monitoring of real time biological interactions (Robinson *et al.*, 1993). Presently the commercial exploitation of SPR has led to the development of the Pharmacia BIAcore™ system which represents a significant breakthrough in immunosensor technology (Byfield and Abuknesha, 1994). The BIAcore™ SPR system incorporates a gold surface which is linked to a carboxymethylated dextran matrix by a hydroxyalkyl thiol layer (Lofas and Johnsson, 1990), Figure 2.9.

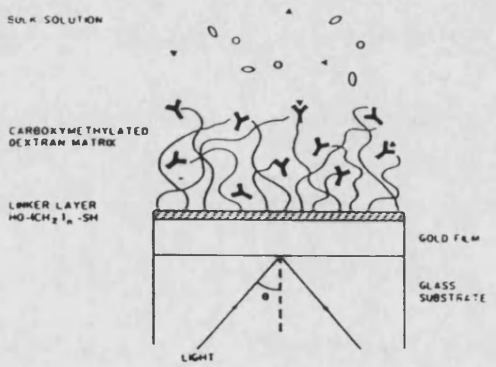


Figure 2.9.

The matrix reduces non-specific binding (NSB) by its hydrophilic nature and promotes biological binding reactions enabling the determination of important clinical samples such as theophylline (mM) and β2-micro globulin (1.7-83nM) to be detected in whole serum at reaction times of 5 min and 8 min respectively. The system is also finding much use as research tool in epitope mapping (Johne *et al.*, 1993).

Other available uses of SPR technology include the detection of Human Chorionic Gonadotrophin (HGC) using a multilayer biotin-avidin-antibody system which has a detection limit of 0.1 nM (Leech, 1994) and an SPR-based immunosensor developed by Amersham plc. employing antibodies labelled with latex beads. These labelled antibodies serve to amplify the changes in refractive index properties of the sensor-surface and, in effect, reduce signal-to-noise ratio.

However although the use of SPR immunosensing technology offers exciting prospects for the direct detection of receptor-ligand binding interactions and is simpler to use than planar optical waveguides, SPR still suffers from a high degree of NSB to the metal surface by proteins in whole serum. It also has a lack of sensitivity comparable to ELISA technology (Leech, 1994) with theoretical analysis comparing the principles and sensitivities of both planar waveguide and SPR sensors predicting higher sensitivities for the waveguiding techniques (Buckle *et al.*, 1993).

2.3 The fluorescence capillary fill device (FCFD)

The need for rapid, simple and easy to use homogenous immunosensors had led to an increase in sensors exploiting the evanescent field and is currently dominating optical biosensing (Narayanaswamy, 1991). Evanescent wave immunosensors have been incorporated into both direct and indirect format and allow surface bound components to be sensed without the need for a separation or washing step.

A number of research groups have incorporated evanescent wave immunosensors into fluorescent immunoassays in both planar optical waveguides and fibre optics, thereby achieving the required sensitivities in serum and whole blood (Sloper *et al.*, 1990, Deacon *et al.*, 1991).

One such optical immunosensing device under commercial development is the fluorescence capillary fill device (FCFD), a fluorescence based evanescent immunosensor developed by Serono Diagnostics. This incorporates a novel capillary fill design (Badley *et al.*, 1987). The system consists of two sheets of glass separated by a narrow gap (100 μm), with the base plate containing an immobilised layer of antibodies and acting as an optical waveguide (Figure 2.10).

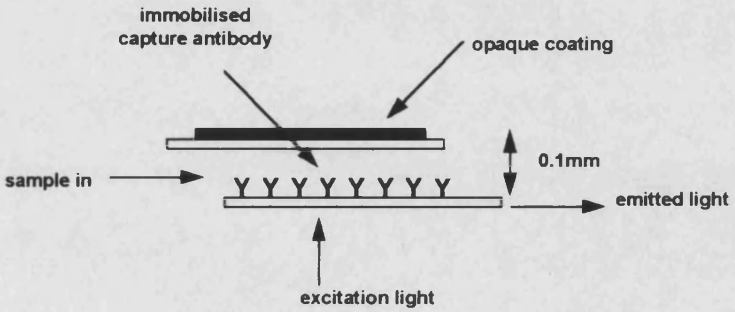


Figure 2.10. The fluorescence capillary fill device (FCFD

The device benefits from the capillary fill system and operates by drawing a fixed volume of sample into the space between the plates, regardless of bulk sample volume.

The Serono immunosensing system contains all, or some, of the reagents required for the assay which can be either covalently coupled or deposited in a soluble releasable form within the device (Parry *et al.*, 1990, Fletcher *et al.*, 1993).

The upper plate, therefore, contains on its surface a layer of fluorescently labelled analyte (antigen) which competes with any unlabelled analyte present in the sample for a limited number of antibody binding sites on the waveguide solid phase (Badley *et al.*, 1987, Parry *et al.*, 1990), Figure 2.11.

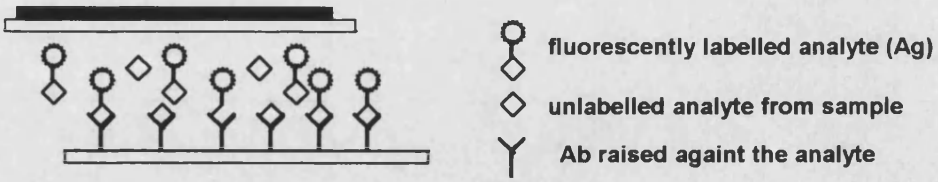


Figure 2.11. Schematic diagram of a competitive binding assay within the FCFD.

The signal is generated by illuminating the device with light of wavelength necessary to excite the fluorophore, normally fluorescein at 415 nm, which results in labelled analytes fluorescing at longer wavelengths. Subsequently, reagent molecules that remain in solution can only fluoresce into relatively large angles relative to the plane of the waveguide in accordance with Snell's law of refraction. However, reagent molecules bound on, or close to, the surface of the lower plate will emit light into a broader range of angles than would be possible from solution and will emerge at angles less than 47° to the plane of the plate (Badley *et al.*, 1987), Figure 2.12.

This coupling into small angles is therefore known as evanescent-field coupling (El-Hang Lee *et al.*, 1979) and allows the detection of surface bound fluorescence without negation of a separation or washing step.

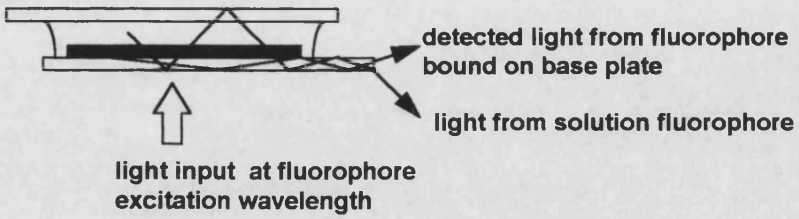


Figure 2.12. Schematic diagram of ray paths of emitted fluorescent light in the FCFD.

The FCFD competitive immunoassay system can be applied to sandwich assays, however the most promising results have been attained with competitive assays with sensitivities of 3.3×10^{-12} M reported for the detection of prostate specific antigen (PSA) (Fletcher *et al.*, 1993). This high sensitivity allied to the use of a whole blood samples, the ease of fabrication at low cost together with calibration of the system, suggest commercialisation of such sensors is not too far away (Robinson, 1993).

CHAPTER 3

PURINE CHEMISTRY

3.1 Introduction

The elucidation of the purine ring system by Fischer, 1898 in the early part of the nineteenth century led the way forward in establishing purine chemistry as one of most important subdivisions of modern bio-organic chemistry. Through his unambiguous synthesis of uric acid (Figure 3), Fischer was able to elucidate its structure which had previously been predicted by Fittig, 1889 and Medicus, 1890 (Lister, 1971).

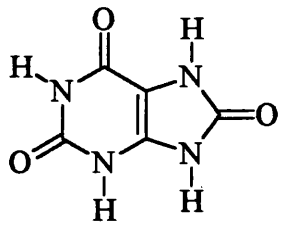


Figure 3. The structure of uric acid

Subsequently, Fischer and co-workers demonstrated the conversion of uric acid to a number of naturally occurring purines (Figure 3.1.), including xanthine from gallstones, guanine from the excreta of sea birds and the N-methylated xanthines, including theophylline and caffeine from beverage plants. Therefore, following Fischer's groundbreaking work, purine chemistry flourished and, in 1953, resulted in the elucidation of the structure of DNA (Watson and Crick, 1953), in which the purines adenine and guanine were constituent bases of the double helix.

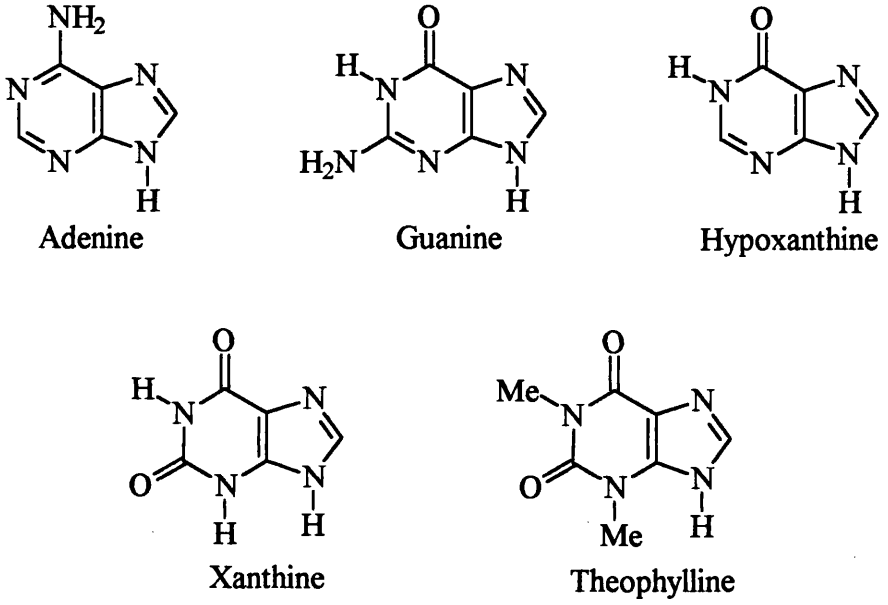
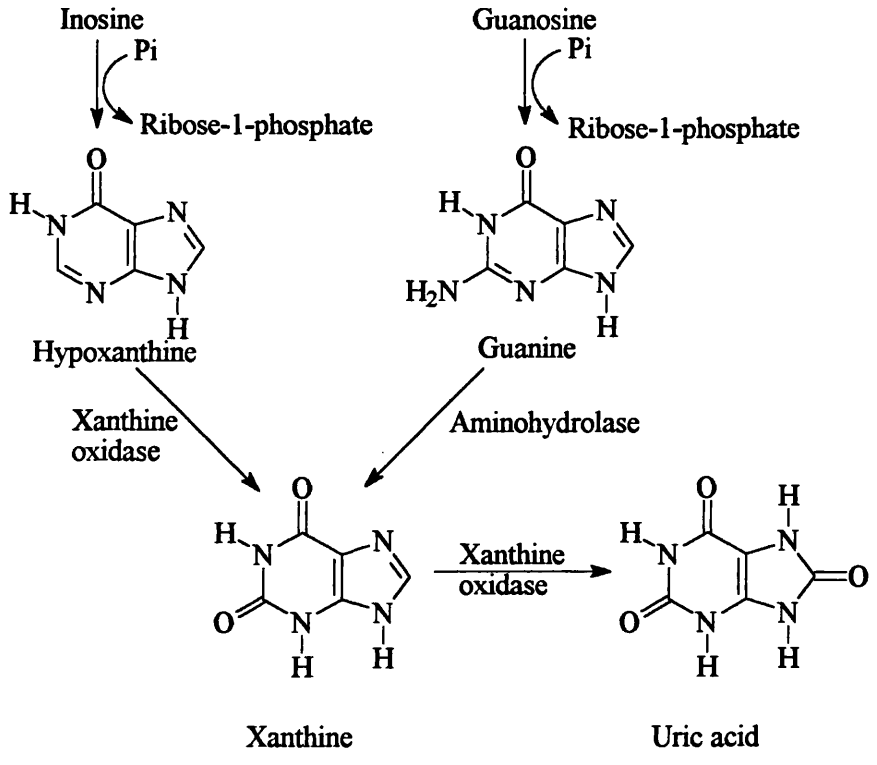


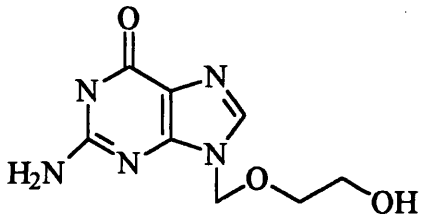
Figure 3.1. The structure of some naturally occurring purines

Since nucleic acids are inherent to all living cells, purines have been detected in a diversity of living matter including the metabolic breakdown products of nucleotides and as vitamin cofactors (Scheme 3).

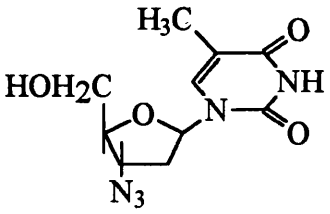


Scheme 3. The catabolism of purine nucleotides

In recent years, understanding of purine chemistry has led to increasing amounts of research being focused on the design of anti-metabolites (e.g. methotrexate) which interfere with nucleic acid replication in malignant cells, and more recently, on antivirals in particular, drugs against HIV and herpes infection which include acyclovir, 6-mercaptopurine and recently AZT (Figure 3.2).



Acyclovir



AZT (3'-azido-2',3'-dideoxythymidin

Figure 3.2. New drugs in anti-viral therapy

3.2 The chemistry of the purine ring

Many of the reactions undergone by purines can be explained by understanding the fundamental physicochemical nature of the heterocyclic system. The purine ring (Figure 3.3.) is basically composed of two heterocycle components, the pyrimidine and the imidazole systems. The pyrimidine component is an example of a π-electron deficient system due to the presence of the N-1 and N-3 nitrogens which provide an electron localising effect within the ring. The imidazole ring structure, containing both singly and doubly bound nitrogens at positions N-7 and N-9, respectively, represents a π-electron excessive system.

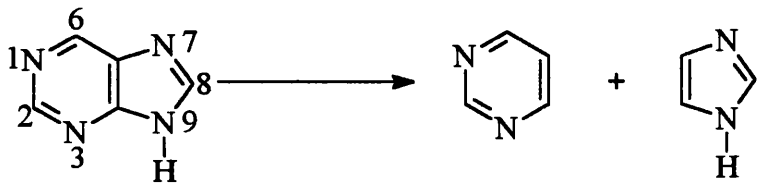
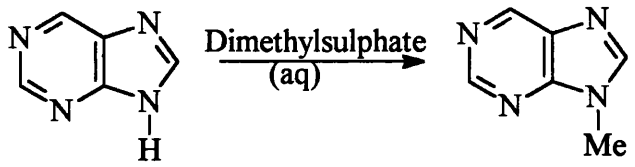


Figure 3.3. The purine ring system

These contrasting electronic systems present within the unsubstituted purine ring result in a sharing of the imidazole π electrons by the pyrimidine moiety and provide a relatively stable bicyclic system. This balance, however, can be disturbed by insertion of the appropriate electron donating or withdrawing groups into either ring and therefore, allows the purine ring to undergo both electrophilic and nucleophilic reactions.

As a consequence of this localisation around the ring nitrogens, the adjacent carbon atom C-8 also shows some degree of electrophilic and nucleophilic character which is not only dependent on the ionisation state of the molecule but also on the type of substituents at the ring carbons.

Subsequently, in the unionised molecule, the carbon at position 8 (C-8) appears to be the most electron-deficient. Substituents at carbon are displaced by nucleophiles in the order C-8>C-6>C-2. However, proton loss within the imidazole ring leads to formation of the anion and an increase in electronegativity, resulting in nucleophilic attack directed initially at C-6 of the pyrimidine ring with subsequent displacement at C-2 and C-8 positions. This anionic form is also readily attacked by electrophiles including alkylating and glycosylating agents to produce normally 9-substituted derivatives (Scheme 3.1).

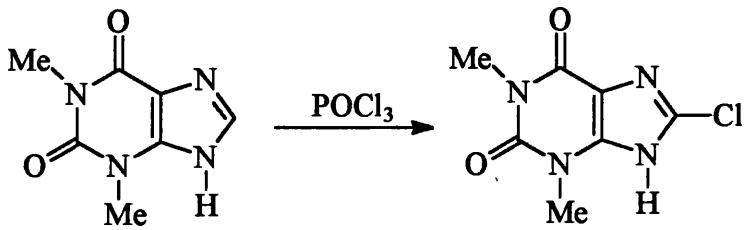


Purine 9-methylpurine

Scheme 3.1. Alkylation of purine in the anionic form

In neutral conditions electrophilic attack results in a mixture of products but the general trend is towards substitution at the 9 position of the molecule although reports of 7 substituted purines do appear in the literature (Shamim *et al.*, 1989).

However, once a ring proton is replaced by an alkyl group, further alkylation is possible and can result in the formation of quaternary salts at either nitrogen in the pyrimidine or imidazole system. Furthermore the introduction of one or two strong electron-releasing groups (hydroxy or similar) into the pyrimidine ring activates the 8-position which may achieve sufficient electronegativity to be attacked by electrophiles. Such electrophilic displacements result in direct alkylation, nitration and halogenation of the 8-position of the purine moiety (Figure 3.4.)



8-chlorotheophylline

Figure 3.4. The direct chlorination of theophylline

3.2.1 Reactions of the purine ring

As described in the earlier section, the reactions undergone by purines are not only governed by the presence of the appropriate electron donating or withdrawing groups but also the state of ionisation of the purine molecule at time of reaction. The majority of purine metatheses in the literature (Lister, 1971) are focused towards nucleophilic displacement reactions usually involving displacement of a halogen or thio group at the 2-, 6- and 8-positions by a wide range of substituents.

Chloropurines are the most versatile derivatives with replacement of the chlorine atoms by nucleophilic reagents governed by the ionisation state of the purine. However, the presence of strong electron-releasing groups leads to electrophilic substitution at the 8-position of the purine moiety and is particularly useful for reactions involving the methylxanthines. These methylated dioxopurines possess two strong electron-releasing groups and can undergo alkylation, halogenation and nitration at this 8-position (Lister, 1971).

The next two sections concern both types of displacement particularly reactions involving halogenation and alkylation of both substituted and unsubstituted purines.

3.2.1.1 Alkylation

The purine ring system, as detailed in section 3.1, is the juxtaposition of a pyrimidine with an imidazole ring and, is capable of undergoing both electrophilic and nucleophilic reactions at its ring nitrogens. Thus one of the most important reactions involving the purine ring is the electrophilic substitution of the ring nitrogens by alkylating agents.

The use of alkylating agents has attracted much attention in purine chemistry not only as a commercial route to the synthesis of the therapeutically important methylated xanthines (e.g. theophylline, theobromine) but also in the understanding of the mutagenic, carcinogenic and anti-tumour effects of such agents in biological systems (Lawley and Brookes, 1963). Therefore, as expected from such a ring system the alkylation of the ring nitrogens is complex and attributed to the ionic state of the purine derivative at the time of reaction. Thus the anionic form of purine (Figure 3.5) is readily attacked by such agents as dimethylsulphate/alkali, diazomethane to produce normally and exclusively N-9 substituted derivatives.

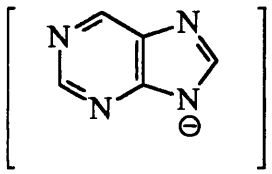


Figure 3.5. The anionic form of purine

However, an excess of methyl iodide in DMF gives the 7,9-dimethylpurinium salt (Figure 3.6) whilst 6-methylpurine gives 6,9-dimethylpurine as the main product.

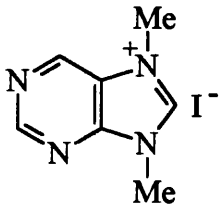


Figure 3.6. 7,9-dimethylpurinium salt

In neutral forms, the products of alkylation may vary although, once the ring proton has been replaced, further alkylation can occur to produce quartenary salts at either the imidazole or pyrimidine ring nitrogens.

Thus, adenine gives mainly the 3-alkylated product under neutral conditions in the presence of alkyl bromide in dimethylacetamide while the anionic form gives a mixture of 3- and 9-alkyl derivatives, although in few cases the 9-isomer predominates (Lister, 1971), Figure 3.7. In contrast, with adenosine and its, derivatives the 1-alkylated product is observed which is attributed to the hindrance to N-3 attack by the *peri* 9-ribose substituent within the DNA helix.

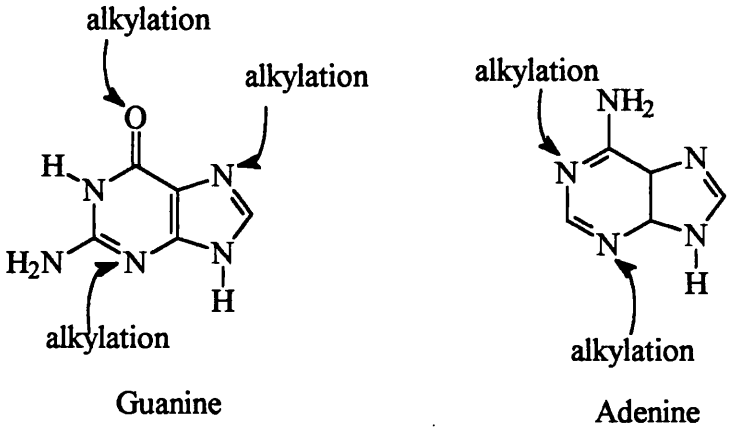


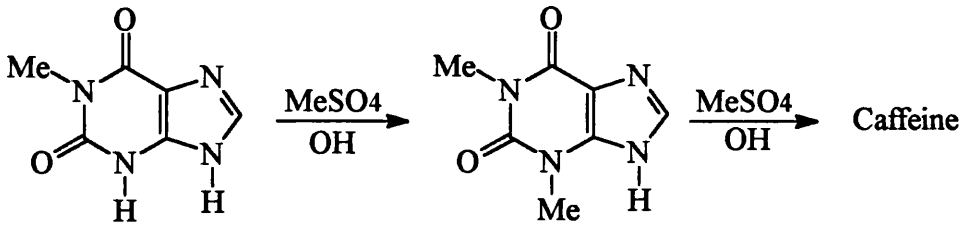
Figure 3.7. The alkylation of the purine bases

Subsequently, in the case of the oxo, thio, thioxo and aminopurines, the availability of additional tautomeric forms enlarge the possibility of attack by electrophiles at the different ring nitrogens.

Thus, alkylation of guanine is a much more complex process which, under alkaline conditions, results in a mixture of 9-, 7- and 3-methyl derivatives. In contrast, the 7-position is readily alkylated when guanine is held in place in the Watson-Crick double helix in nucleic acids, by such alkylating agents as N-nitroso-N-ethylurea, dimethyl sulphate (DMS) and diazomethane (Strauss, 1976, Singer and Kusmierek, 1982).

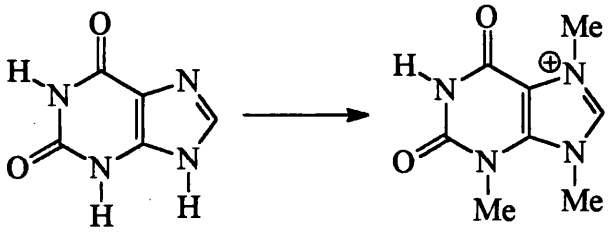
This preferential site of alkylation is attributed to the availability of the N-7 position for reaction with electrophilic reagents and being the most nucleophilic centre within this conjugated system (Lawley and Brookes, 1963). Further alkylation at this position gives rise to an unstable quaternary nitrogen which leads to separation of the purine ring from the deoxyribose, unit leading to interference in DNA replication (Hall, 1971).

Thus, extending this system to the alkylation of oxygenated purines, in particular to xanthine and its derivatives, it has been observed that alkylation under alkaline conditions proceeds in the order N-3, N-7, N-1 (e.g. in the order of decreasing acidity) with methylation of xanthine giving first theobromine then caffeine, in the presence of methyl iodide and barium carbonate. Similarly 1-methylxanthine is methylated by dimethyl sulphate in alkaline solution to furnish 1,3-dimethylxanthine (theophylline) which is further alkylated to caffeine (Scheme 3.2)



Scheme 3.2.

However, blocking of oxygen function by trimethylsilylation leads to exclusive alkylation at N-7. In contrast, under neutral or acidic conditions, methylation of xanthine proceeds at the N-7 or N-9 position. Thus, in the presence of methyl iodide 7,9-dimethylbetaine is formed via reaction with xanthine (Scheme 3.3) while theophylline and analogues methylate exclusively at N-7 and theobromine at N-1.

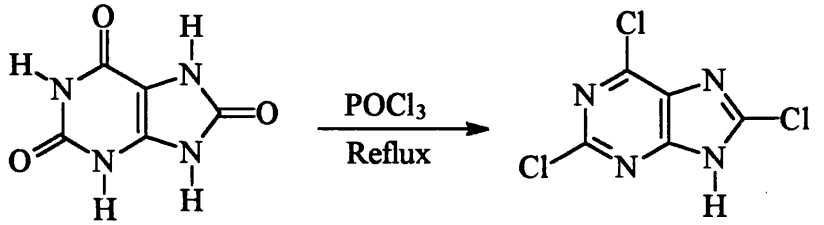


Scheme 3.3. The formation of 7,9-dimethylbetaine

3.2.1.2 Chlorination

Chlorination of the purine moiety is one of the most important reactions in purine chemistry and provides a route to both substituted and unsubstituted derivatives of purines. Subsequently, the exceptional reactivity of the chloropurines toward nucleophilic reagents makes them invaluable compounds for purine inter-conversion. Therefore, chlorine atoms located at the 2-, 6-, or 8-position are all replaceable but the ease or difficulty of displacement is modified in the presence of electron-releasing groups (Lister, 1971).

The synthesis of chloropurines has been achieved by a variety of methods of which the most successful has involved the inter conversion of appropriate oxo analogues to their corresponding 2-, 6- and 8-chloro derivatives. This transformation is normally carried out using a phosphorous halide, such as phosphorus oxychloride ($POCl_3$) either alone or in a mixture with phosphorous pentachoride (PCl_5) in an inert solvent such as toluene. This approach is extensively used in purine chemistry and has been adapted and modified from Fischer's initial synthesis of 2,6,8-trichloropurine from uric acid in the presence of phosphorus oxychloride (Lister, 1971), Scheme 3.4.

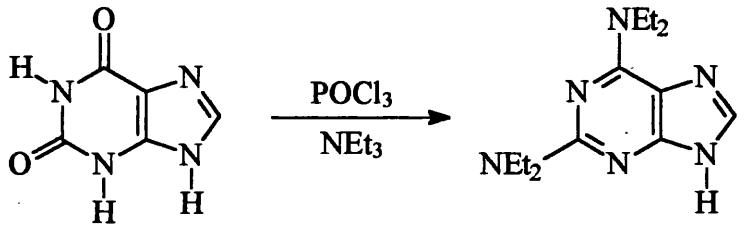


Uric acid 2,6,8-trichloropurine

Scheme 3.4. The synthesis of 2,6,8-trichloropurine

Adaptations of this method are extensive in the literature (Lister, 1971, Finar, 1981) and normally employ the combination of phosphorus oxychoride with phosphorous pentachloride used under reflux to synthesise the appropriate chloro derivatives from their corresponding di and trioxo counterparts.

However, a more efficacious procedure involves the use of tertiary aliphatic or aromatic amines (e.g. N,N-dimethyl or diethylanilines) as catalysts and/ or acid scavengers in the presence of phosphorus oxychloride for the chlorination of purines such as hypoxanthine to 6-chloropurine which is not achieved by standard conditions. Interaction between the chlorine atom and the tertiary nitrogen atom is rare but some side reactions involving replacements of halides by amine fragments e.g. (1) to (2) or complex dealkylative rearrangements of alkyl derivatives have been reported, Scheme 3.5.



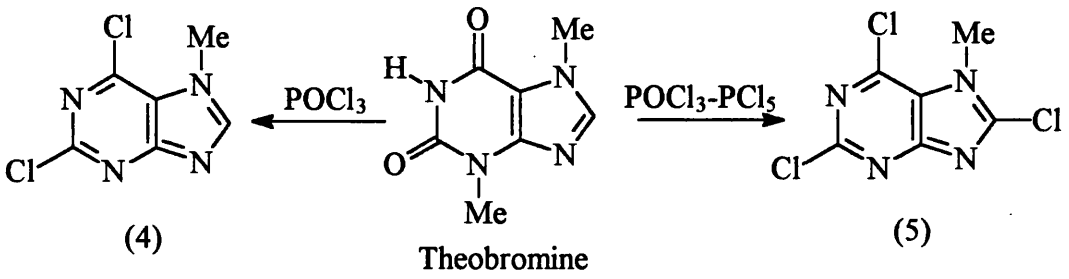
(1) (2)

Scheme 3.5.

Even nucleosides may be chlorinated by this combination of reagents, providing that the sugar hydroxy groups are protected as acetoxy or benzyloxy groups. Other reagents which have been used include thionyl chloride in DMF (Vilsmeier-Haack reagent) which converts 2',3',5'-tri-O-benzoylinosine into a 6-chloropurine nucleoside, and chloromethylenedimethylammonium chloride in chloroform.

The presence of other groups is not, in general, deleterious to the purine molecule with alkyl groups (also N-alkyl), aryl, trifluoromethyl, amino and alkylthio substituted purines having all been successfully chlorinated. Direct chlorination of the purine nucleus has been achieved primarily through nucleophilic attack on the 8-position and is only possible if the unoccupied 8-position contains one or more electron releasing groups (*e.g.* hydroxy or oxo group), with the course of halogenation affected by the nature of the solvent used.

As a general rule chlorination of N-alkyl oxopurines is only possible in those cases where the oxo function is capable of existing in the enol form. Therefore alkylated oxopurines in which no enolisation is possible may also react but usually with elimination of one or more alkyl groups. Of the methylated forms theobromine (3) allows chlorination of both 2- and 6-positions through loss of N-3 methyl group (Finar, 1981). In the presence of dimethylaniline purine (4) arises, whereas using a mixture of phosphorus oxychloride and phosphorus pentachloride results in the concurrent nuclear chlorination of C-8 giving 7-methyl-2,6,8-trichloropurine (5), Scheme 3.6.

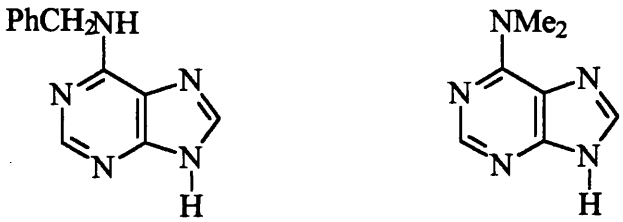


Scheme 3.6.

Similarly caffeine using the above mixture at elevated temperatures (180°C) gave the same trichloropurine (Lister, 1971) while 1,9-dimethylxanthine in phosphorus oxychloride at 140°C gave the corresponding 2,6-dichloro-9-methylpurine. Therefore direct chlorination of N-alkylated oxopurines is achieved by a number of reagents including thionyl chloride in chloroform, chlorine gas and in some cases N-chlorosuccinimide and sulphoryl chloride when 8-methylpurine is used.

The high reactivity associated with halogenopurines as well as their ease of removal by a wide variety reagents infers that the chlorination of the purine moiety is one of the most important features of purine chemistry.

Replacement of chlorine by hydroxyl proceeds with ease under alkaline conditions only if other activating groups are present in the molecule with ring opening of the imidazole ring following hydroxyl attack at C-8, especially when 7 or 9-alkyl groups are present. However the most widely used reactions of chloropurines has been with amines, leading to a wide variety of purines of synthetic and biological importance including N-benzyladenine (6), the base puromycin (7) and other similar adenines, Scheme 3.7.

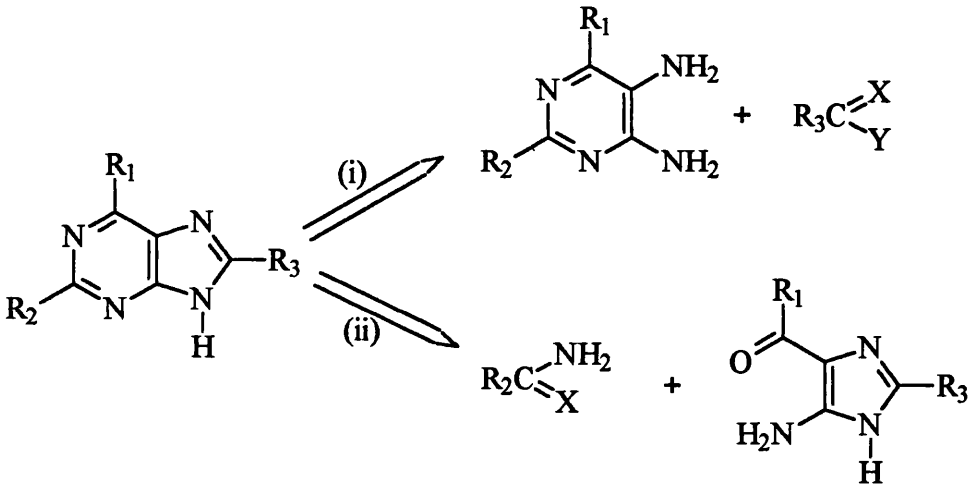


(6) (7)

Scheme 3.7.

3.3 Synthetic routes to purines

The purine ring is the fusion of two aromatic heterocycles, the pyrimidine and the imidazole. A logical starting point for ring synthesis is an appropriately substituted pyrimidine or imidazole molecule from which a second ring can be constructed by a cyclisation process, this is illustrated in Scheme 3.8



Scheme 3.8. Synthetic routes to purines via two pathways

Two methods of synthesis are present in Scheme 3.8 and both have been successful for the synthesis of the purine moiety. The first method (i) is the most common and is known as the Traube route and is by far the most versatile method for the synthesis of purines.

The 5,6-diaminopyrimidine starting compound reacts with a wide range of electrophiles particularly acids, acid chlorides, formamides, ethyl orthoformate and other similar electrophilic reagents to become the intermediate 5-acylamino substituent of the pyrimidine component, which *via* cyclisation with base or phosphorus oxychloride provides the ring-closed 8-substituted purine.

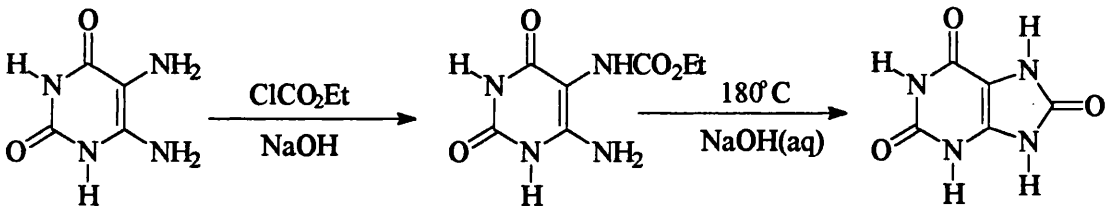
The synthesis *via* route (ii) is a useful alternative to that based on diaminopyrimidines and involves the use of 5-aminoimidazole-4-carboxamide and related compounds as a starting precursors for the purine moiety. The route utilises a variety of reagents commonly used in the cyclisation process of the Traube route and include formic acid, alkyl and trialkyl esters, amides and urea. Intermediates isolated are normallly the formylated or acylated amino compounds of the starting carboxamides which are subsequently ring closed by heating directly or by base catalysis. This method is closely related to the biological route to purines, with the starting 5-aminoimidazole-4-carboxamide occurring naturally as a ribonucleotide and being the precursor of purine nucleotides. Imidazoles are, therefore, useful in the synthesis of both purine nucleosides from imidazole nucleoside precursors and in the synthesis of 2,6,8-substituted purines reflecting the type of carboxylic acid used in the building of the purine ring.

3.3.1 The Traube route

The Traube route to purines is the most widely used and versatile synthetic route to purines. Modifications have led to the synthesis of a wide range of substituted and unsubstituted purines, including the pharmacologically important methylxanthines (Traube, 1900), adenosine antagonists (Jacobson *et al.*, 1988, Peet *et al.*, 1993) and derivatives of theophylline (Pfleiderer and Nübel, 1959, Fuschs *et al.*, 1978, Miyamoto *et al.*, 1993).

In recent years the route has been modified and generalised to two main synthetic procedures both incorporating a pyrimidine precursor.

The first and most commonly utilised starting precursor is 5,6-diaminopyrimidine (Papesch and Schroeder, 1951, Speer and Raymond, 1953) which reacts with carboxylic acids or their corresponding acid chlorides to produce the 5-acyl intermediate with ring closure in aqueous alkali provides a route to purines unsubstituted at the 8-position. The earliest example of the use of such precursors was in the synthesis of uric acid *via* the acylation of the starting diaminouracil (Traube, 1900). Cyclisation of the imidazole moiety was effected by boiling in base, Scheme 3.9.

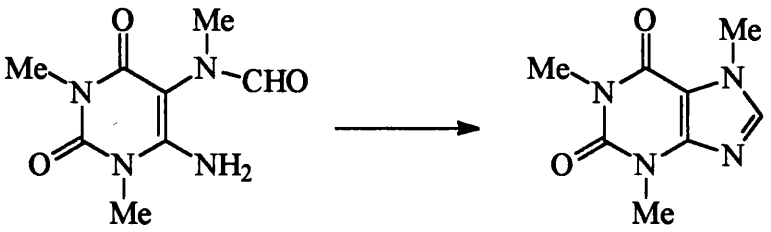


5,6-diaminouracil

Uric acid

Scheme 3.9-The synthesis of uric acid via the Traube route

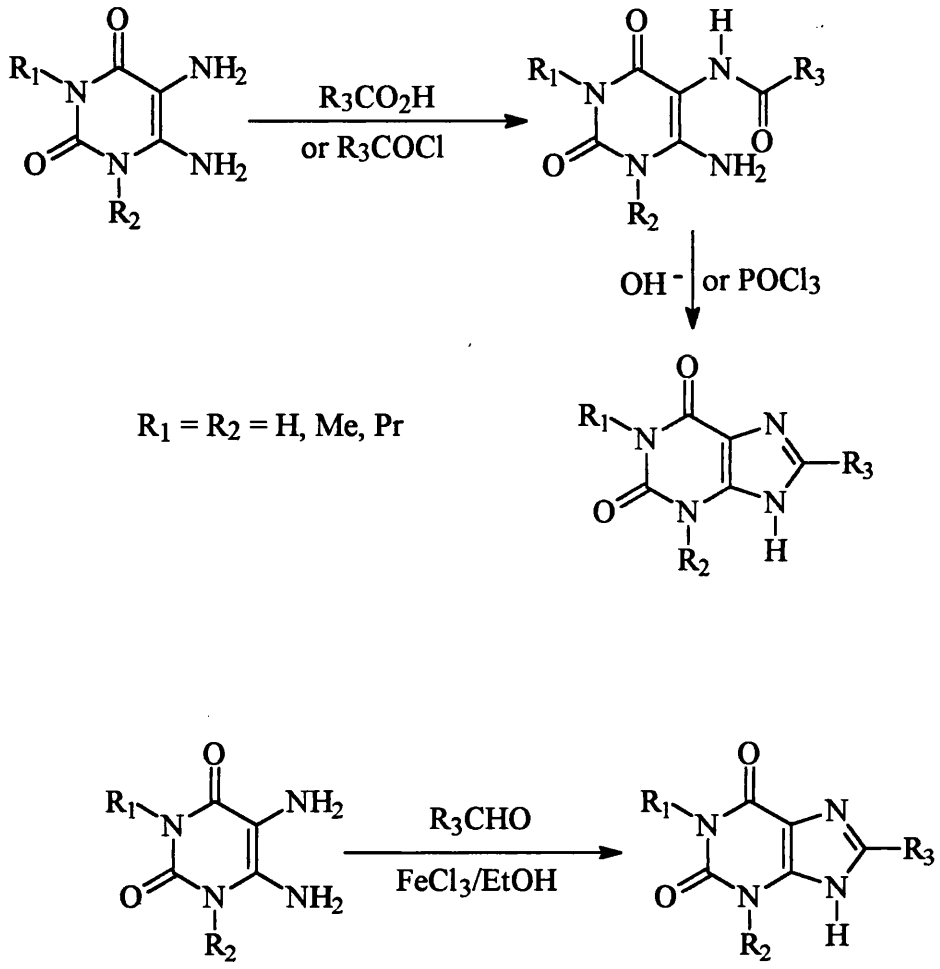
The intermediates during this reaction are usually the N-formyl or N-acyl derivatives of the 5-position, with acylation/formylation through reaction with formic acid-acetic anhydride mixture or with formamide alone (Traube, 1900). Cyclisation of these intermediates is through fusion or heating in alkali with ease of ring closure associated with the acidity of the 5-formamido intermediate as outlined by the spontaneous cyclisation of the weakly acidic methylated pyrimidine to caffeine (Scheme 3.10).



Caffeine

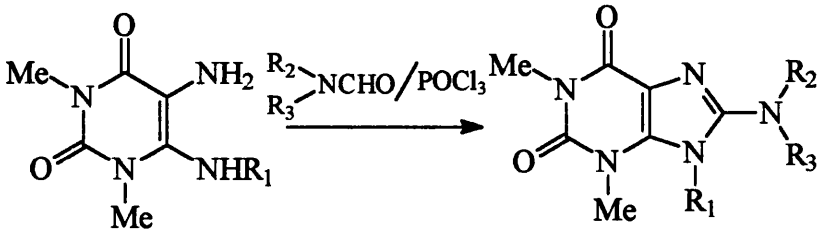
Scheme 3.10. The cyclisation of intermediate 5-formamido derivative to caffeine

Subsequent extension of this route to 8-substituted purines has close parallels with methods for unsubstituted derivatives with the starting diaminopyrimidine precursor being acylated with the corresponding substituted acids or their acid chlorides. Ring closure, although more difficult with such intermediates, can alternatively be effected either through treatment with alkali or refluxing with phosphorus oxychloride (Traube, 1900, Shimada, 1992), Scheme 3.11. Further, condensation of the diaminopyrimidine with an aldehyde, followed by oxidative cyclisation, also gives the corresponding 8-substituted product, particularly 8-alkyl and 8-aryl derivatives (Traube, 1900), Scheme 3.11.



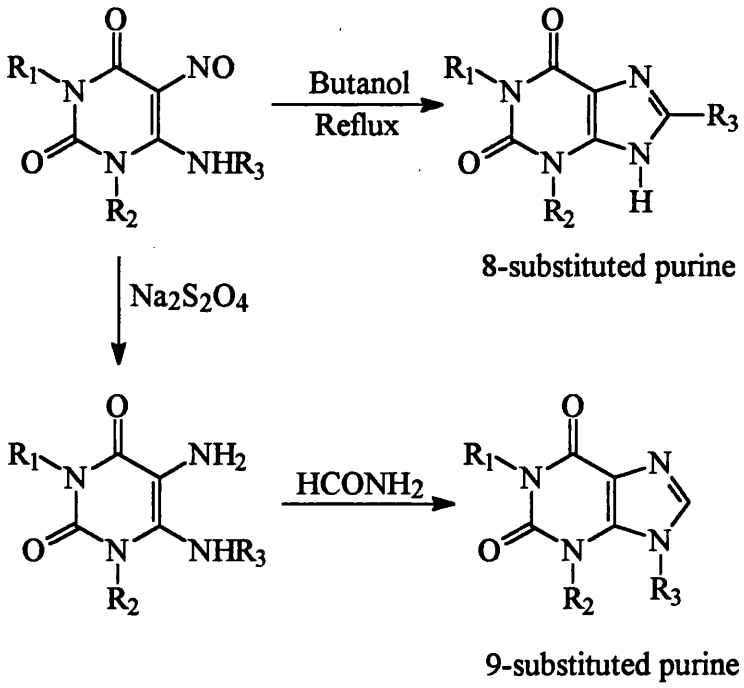
Scheme 3.11. Routes to 8-substituted purines

Adaptations to Scheme 3.11 include, the synthesis of 8-amino derivatives of theophylline following the reaction of 5,6-diaminopyrimidine with a mixture of DMF in phosphorus oxychloride (Vilsmeier-Haack reagents). Recent modification of this procedure by Yoneda and co-workers utilising the 5-nitroso-4-amino precursor (1973, 1974) have led to the *insitu* condensation and cyclisation of the precursor to 8-methylaminotheophylline, Scheme 3.12.



Scheme 3.12. Synthesis of 8-aminopurines using Vilsmeier-Haack reagents

Routes to 8-substituted purines although successful through utilisation of the starting diaminopyrimidine precursor have been superseded by cyclisation of the 5-nitroso-4-alkylaminopyrimidines. Nitrosation of the 5-position affords derivatives which can functionalised to afford both 8- and 9-substituted purines, Scheme 3.13.



Scheme 3.13 Routes to 8-and 9-substituted purines

Cyclisation under refluxing conditions in butanol or xylene has been developed through the work of Bredereck *et al.*, (1962), Pfleiderer and Walter, 1964, and Goldner *et al.*, (1966). This cyclodehydration reaction utilises the reaction between the C4-alkylamino and the 5-nitroso group with the loss of water.

Alternatively, reduction of this nitroso derivative in a mixture of formamide/formic acid results in concurrent cyclisation to the 9-substituted derivative (Pfleiderer and Nübel, 1960). Similarly addition of sodium nitrite followed by heating in sodium dithionite at low temperatures also gave the 9-substituted derivative, Scheme 3.13.

3.3.2 Synthesis of purines from imidazoles

Although the most common route of synthesis for purine analogues has been *via* the use of a pyrimidine precursor, the use of other alternative intermediates has been investigated with the use of imidazole derivatives being popularised by many researchers in the purine field as appropriate starting products for synthesis of a wide variety of purines.

The first synthetic approach to purines *via* an imidazole intermediate was due to Sarasin and Wegmann in 1924, (Lister, 1971). However, the lack of suitable intermediates together with the lability and oxidation of these derivatives under normal conditions made them difficult to work with. In recent years, however, these disadvantages have been overcome with imidazole derivatives providing not only a suitable means by which substituted and unsubstituted derivatives of purines could be synthesised but also offering a route to purine nucleosides and nucleotides under milder reaction conditions.

Nowadays purine syntheses from imidazoles involve the use of the starting intermediate 5(4)-amino-4(5)-carboxamide and its related compounds including the thiocarboxamide (8), carboxamidine (9), nitrile (10) and the corresponding ester (11) (Figure 3.8).

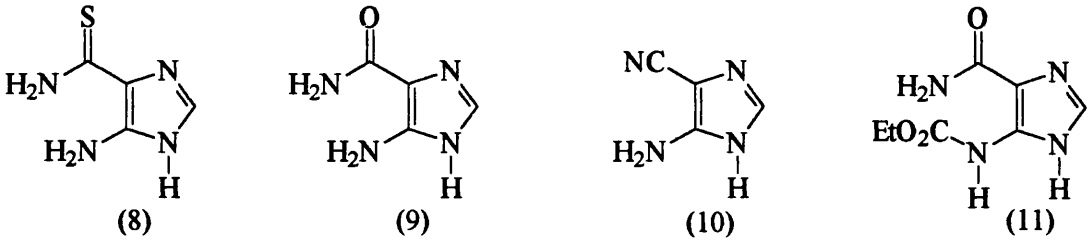


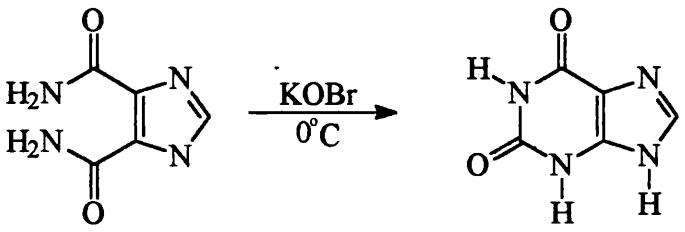
Figure 3.8. Imidazole derivatives for use in purine synthesis

The intermediates used for the completion of the purine ring are much the same as for the cyclisation of diaminopyrimidines involving the use of formic and carbonic acids but requiring much milder conditions. Therefore, the use of imidazole precursors has provided a useful route to purines not possible by other conventional methods.

In the next two sections the use of 4-amidoimidazole-5-carboxamide and 5(4)-aminoimidazole-4(5)-carboxamide as precursors to purines particularly substituted purines, including xanthine and its derivatives, will be reviewed.

3.3.2.1 The use of 5-aminoimidazole-4-carboxamide as a precursor

The use of 5-aminoimidazole-4-carboxamide for the preparation of xanthine and its derivatives was first observed by Baxter and Spring 1944 (Lister, 1971) using potassium hypobromite *via* the Hofmann degradation of 4,5-diaminocarboxamide (Scheme 3.14).



Xanthine

Scheme 3.14.

The following year Baxter and Spring, 1945 (Lister, 1971) expanded this route to the synthesis of 9-alkylxanthines (13) by use of the appropriate N-alkylimidazole precursor (12, R=alkyl) via the Lossen rearrangement of the 4-carboxamide group with hydroxamic acid, Figure 3.9.

(12) (13)

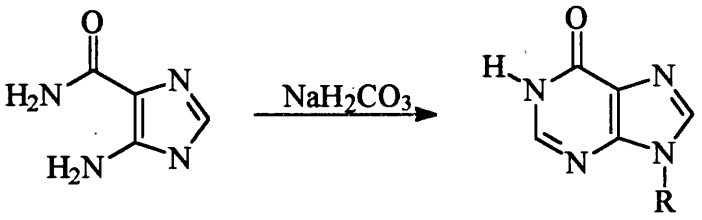
Figure 3.9

As a result of this early work this procedure has found use not only in the preparation of xanthine derivatives, but also as a possible route to nucleosides by use of the appropriate starting 5-glycosylimidazole-4-carboxamide.

3.3.3.2 The use of 5(4)-aminoimidazole-4(5)-carboximide as precursors

Almost all recorded purine syntheses from imidazoles involve the starting precursors of 5(4)-aminoimidazole-4(5)-carboximides which under appropriate conditions are cyclised to the purines in question.

Thus the synthesis of hypoxanthine is achieved through the formylation of the starting 5-aminoimidazole-4-carboxamide in the presence of formic acid-acetic acid mixture. The C-2 substituted product is subsequently obtained by treatment of the N-formyl derivative with a mild base, Scheme 3.15.

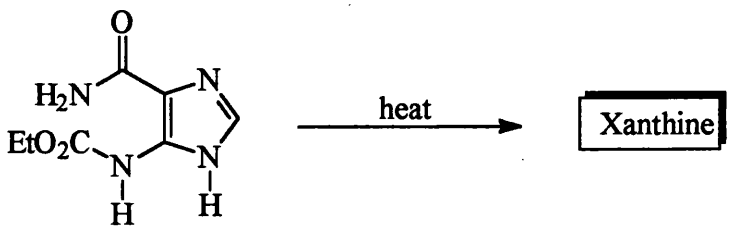


Hypoxanthine (R=H)

Scheme 3.15. Route to hypoxanthine

Contrasting this to the Traube route, formamide has little application as a one carbon cyclisation reagent with amino imidazoles however, 7-methylxanthine was obtained on heating 4-amino-1-methylimidazole-5-carboxamide with formamide.

In an analogous reaction adenine was obtained from the aminoimidazonitrile in the presence of formamide although yields were improved on heating with formamide acetate.



Scheme 3.16 Routes to xanthine

The xanthines are prepared by reaction of 5-aminoimidazole-4-carboximide with diethylcarbonate in refluxing sodium ethoxide, scheme 3.16.

This route has been readily expanded to accommodate the preparation of nucleoside derivatives including the synthesis of xanthosine via use of 5-aminoimidazole-4-carboximide riboside and 6-thioxanthosine similarly from 5-aminoimidazole-4-thiocarboximide riboside.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The development of a novel one-step homogeneous immunoassay for theophylline is an important goal in both clinical chemistry and diagnostics. Current methods for the measurement of theophylline serum concentrations rely on heterogeneous systems and include such techniques as ELISA (Danilova and Vasilov, 1991), radioimmunoassay (Neese and Soyka, 1977), enzyme immunoassay (Eppel et al, 1978, Rupchock *et al.*, 1985) and fluoroimmunoassay (Meola *et al.*, 1979). These methods although both specific and sensitive do not fulfil the criteria for a rapid, easy-to-use and cost effective assay. Recently focus has moved towards immunosensors which not only offer speed and convenience to the user but require no separation step.

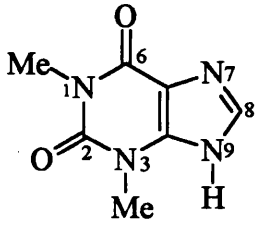
One such technology which has shown great promise is the FCFD a homogeneous assay system (Badley *et al.*, 1987), based on optical immunosensing, which provides an easy-to-use assay with a rapid reaction time. In view of the advantages of such a detection system particularly in the assay of small molecules, the FCFD was used to develop an immunoassay for theophylline with the potential of monitoring serum plasma concentrations with the ability of discriminating between toxic and normal levels. However, in the course of constructing a homogeneous assay for theophylline utilising the FCFD, it became necessary to prepare fluorescent analogues of theophylline which were not only immunoreactive against the anti-theophylline antibody, immobilised within the device, but also soluble in the required assay system.

Further, the photoproperties of the chosen fluorophore together with the length and lipophilicity of the link between theophylline and the fluorophore must be optimised prior to use in the competitive theophylline assay. Thus the synthetic approaches utilised in the preparation of such congeners is outlined in section 4.2.

4.2 Synthetic Approaches

The main aim of the synthetic procedure was to synthesise 8-substituted derivatives of theophylline containing a hydrophilic linker. These linkers would incorporate either an alkylamino or an alkylethoxy chain and possess a terminal functional group (*e.g.* amino or carboxy) which could be used as an attachment point in linking the fluorescent group to the theophylline moiety. However prior to synthesis of such derivatives a number of approaches were considered and are subsequently outlined:

(i) the first and most important concern was the attachment point of the fluorescent group to theophylline moiety. As is apparent in Figure 4, theophylline can be conjugated to a large protein complex or a linker molecule via positions 3, 7, 8 or 9.



Theophylline

Figure 4. The structure of theophylline

Subsequently, in view of these available positions, several groups (Neese and Soyka, 1979, Danilova, 1991) have utilised the 8-position of theophylline in the production of monoclonal antibodies by synthesising conjugates attached to the 8-position of theophylline. While others have seen highly immunogenic derivatives by utilising the 3 and 9-positions (Cook *et al.*, 1976, Hu *et al.*, 1980). However since the attachment point corresponding to the supplied monoclonal antibody was unknown, the 8-position was chosen as the most useful option for the synthesis of the required compounds.

(ii) Once the position of attachment of the fluorescent probe to theophylline was established, the route of synthesis to such derivatives was discussed. There were two possible options available:

(a) involving the synthesis of the theophylline moiety from the starting pyrimidine *via* modification of the Traube route
(b) by use of an appropriate imidazole precursor.

The utilisation of these two routes has been outlined in section 3.3 with both procedures being prevalent in literature (Lister, 1971). The route chosen for the synthesis of 8-substituted theophyllines was therefore, *via* the starting pyrimidine which provided both scope and versatility in the final synthetic approach.

Initially two routes were investigated: through ring closure of the starting 5,6-diaminopyrimidine (A) and the cyclodehydration of the appropriate 5-nitroso-4-amino-1,3-dialkylpyrimidine (B), Figure 4.1.

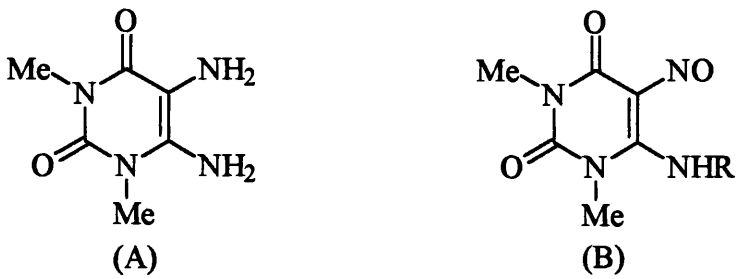


Figure 4.1. Pyrimidine precursors to 8-substituted theophyllines

The use of the starting diaminopyrimidine involved a one-step procedure to the ring closed theophylline derivative. The route followed initial acylation of the 5-amino group followed by ring closure in the appropriate solvent. Conditions generally favoured were refluxing of the acyl intermediate in formic acid, formamide, or through base catalysed cyclisation (Bredereck and Föhlisch, 1962, Lister, 1971).

Subsequently, the versatility of 5,6-diamino-1,3-dialkylpyrimidines has been utilised as starting precursors for the synthesis of a wide variety of 8-substituted purines including derivatives of theophylline (Speer and Raymond, 1953, Bredereck and Föhlisch, 1962, Goldner *et al.*, 1966), adenosine antagonists (Daly *et al.*, 1982, Jacobson *et al.*, 1988, Katsushima *et al.*, 1990, Suziki *et al.*, 1992, Peet *et al.*, 1993) and PDE inhibitors (Kramer *et al.*, 1977, Buckle *et al.*, 1993).

The second route employed involved the use of 5-nitroso-1,3-dimethyl-4-alkylaminopyrimidine which through heating in butanol or xylene provides a route to the desired 8-substituted theophylline product. Subsequently, cyclisation through this procedure has been associated with a number of research groups including Bredereck and co-workers (1962) and Goldner *et al.*, (1966).

(iii) Once the route of synthesis was established the choice of linker was then sought. The synthesis of 8-substituted theophyllines were required to possess a functional terminal amino or carboxy group.

Such groups were chosen due to their ease of formation and versatility for inter group conversion particularly in their reaction with electrophiles including isocyanates and isothiocyanates. These groups are present in the most common fluorescent labels (*e.g.* fluorescein, rhodamine) and are particularly useful in the linkage of the theophylline moiety to the fluorescent moiety.

Thus the initial synthetic strategy involved the synthesis of 8-carboxytheophylline via the oxidation of 8-hydroxymethyltheophylline with possible extension to 8-(3-carboxypropyl)theophyllines.

(iv) The choice of the fluorescent congener was determined prior to synthesis of the 8-substituted theophyllines. The FCFD had already been used in a rubella assay (Parry *et al.*, 1990) in which the label employed was fluorescein which was attached as its isothiocyanate (FITC). Other probes considered for attached to the theophylline moiety were DTAF (dichlorotriazinylfluorescein) and dansyl chloride.

The aim of the synthetic procedure was to synthesise fluorescent probes of theophylline possessing a hydrophilic spacer between the 8-position of theophylline and the fluorescent label. The spacer would incorporate a 5-10 carbon unit between the two functional groups and would contain either a polymethylene or a polyether group as the linker (Figure 4.2).

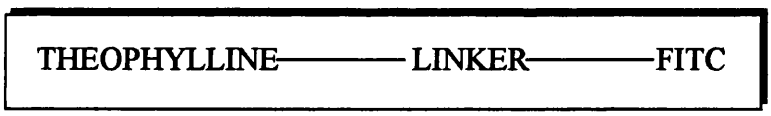


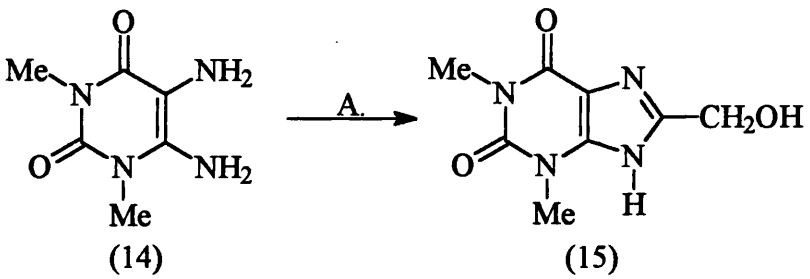
Figure 4.2. The required analogue of theophylline in the FCFD assay

Ultimately the strategy was to synthesise fluorescent probes of theophylline which were not only soluble in alkaline buffer solutions as well as biological samples but be immunoreactive towards the anti-theophylline antibody. This was the most important requirement, since the basis of the immunoassay is competition between the labelled theophylline moiety and unlabelled theophylline.

4.3 The synthesis of 8-carboxytheophylline

As outlined in the previous section the requirement for a suitable theophylline derivative for the attachment of a fluorescent probe was the main priority of the synthetic work. Subsequently, initial work focused on the synthesis of 8-carboxytheophylline (20) from the oxidation of 8-hydroxymethyltheophylline precursor (15). Direct routes to 8-carboxytheophylline have not been reported therefore, functionalisation of the hydroxy precursor to the carboxy moiety was considered to be the most suitable approach for obtaining (20).

However, investigation into the synthesis of 8-hydroxymethyltheophylline led to two routes being considered. Initial work focused on the one step procedure of Bredereck and Föhlisch (1962) through the acylation and cyclisation of the starting 5,6-diamino-1,3-dimethylpyrimidine precursor (14), Scheme 4.



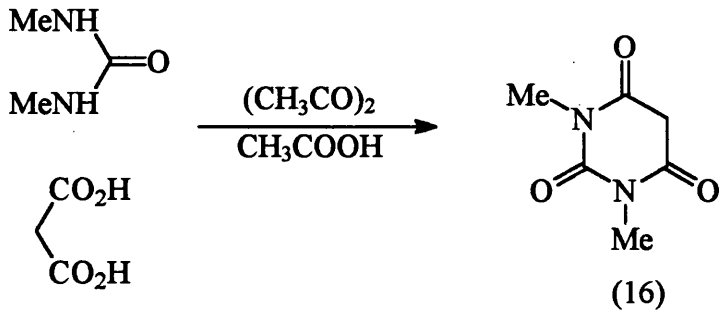
(14) (15)

Scheme 4. A. Hydroxyacetic acid, NaOH.

Acylation of the starting 5,6-diamino-1,3-dimethylpyrimidine (14) was primarily through reaction with hydroxyacetic acid following *insitu* formation of the 5-acyl intermediate which following cyclisation in base afforded the hydroxy derivative (15) in very low yields (2.6%).

Isolation of this cyclised (15) product was through recrystallisation in water, with analysis of proton spectrum confirming the presence of the two N-Me groups of the theophylline moiety which resonate as singlets at δ 3.25 and δ 3.40 together with a signal at δ 4.75 which was assigned to the purine-CH_2 group.

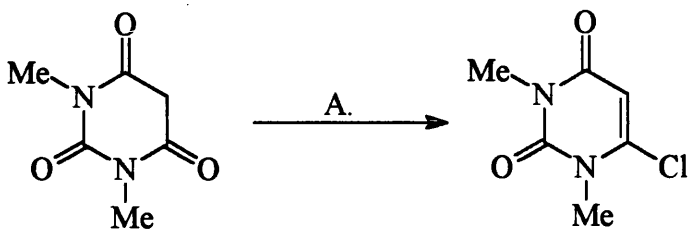
Disappointment in obtaining such low yields of the hydroxy precursor (15) through this one-step procedure led to the preparation of (15) via an alternative route. Thus the synthesis of 8-hydroxymethyltheophylline followed the multi-step procedure of Goldner *et al.*, (1966) involving the initial preparation of 1,3-dimethylbarbituric (16) through condensation of N,N'-dimethyl urea with propane dioic acid in the presence of a mixture of acetic anhydride/acetic acid, scheme 4.1.



(16)

Scheme 4.1. The synthesis of 1,3-dimethyl-2,4,6-trione

Treatment of this pyrimidinetrione (16) with phosphorus oxychloride ($POCl_3$) and a small catalytic amount of water afforded 6-chloro-1,3-dimethylpyrimidine (17) through Scheme 4.2. Removal of excess $POCl_3$ from the final reaction mixture led to the isolation of the chloro compound in 65% yield.

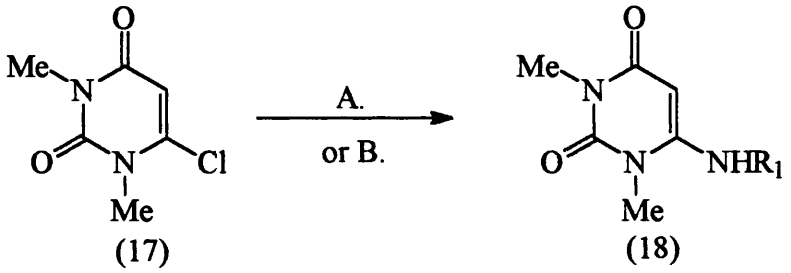


(16) (17)

Scheme 4.2. A. $POCl_3$, reflux, 40 min.

However although the 6-chloro derivative (17) was obtained in high yields there were problems involved in the reproducibility of this derivative in a pure form. The removal of $POCl_3$ from the reaction mixture was problematic as was the possibility of further chlorination of the carbonyls at positions 2 and 4 of the pyrimidine moiety. Subsequently, 6-chloro-1,3-dimethylpyrimidine (17) was obtained commercially and used in the proceeding steps without difficulty.

Displacement of the 6-chloropyrimidine (17) by ethanolamine under reflux afforded 6-(2-hydroxyethylamino)pyrimidine (18) through Scheme 4.3 (Condition A). However isolation of this product by recrystallisation in water proved to be troublesome with final yields as 50% maximum.



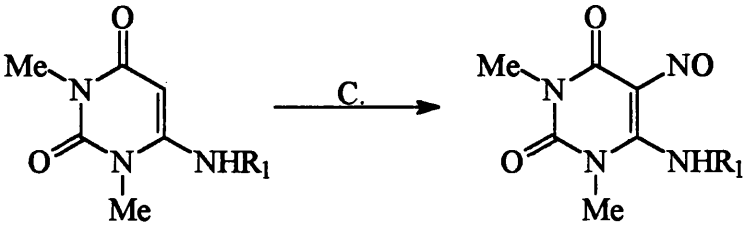
(17) (18)

Scheme 4.3. A. H_2NR_1 / heat $R_1=CH_2CH_2OH$, $R_1=CH_2CH_2CH_2OH$

B. H_2NR_1, Na_2CO_3, heat

Alternatively modification of this procedure, Scheme 4.3 (Condition B), through refluxing of the chloro derivative (17) with ethanolamine in the presence of Na_2CO_3 in ethanol led to the isolation of (18) in 75% yield.

The synthesis of (19) followed nitrosation of the 5-position using isoamyl nitrite in the presence of aqueous acid, Scheme 4.4. The reaction proceeded swiftly and afforded the nitroso derivative (19) as a purple crystalline product in 63% yield. However, it was apparent that prolonging the reaction time of the nitrosation step resulted in the formation of side products, this was confirmed by TLC. Subsequently reaction times were limited to the formation of the purple nitroso product.



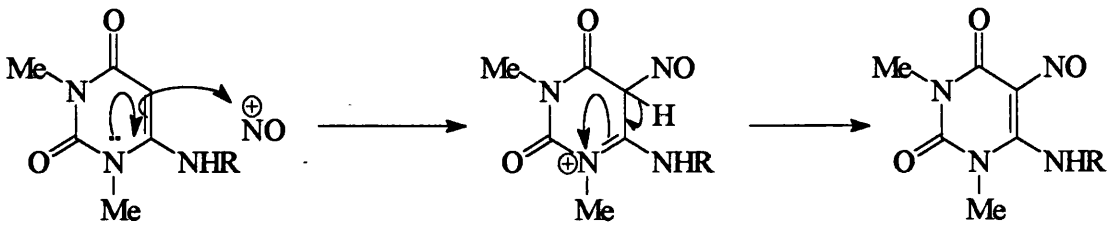
(18) (19)

Scheme 4.4. C. isoamyl nitrite, HCl, EtOH, RT. $R_1=CH_2CH_2OH$

$R_1=CH_2CH_2CH_2OH$

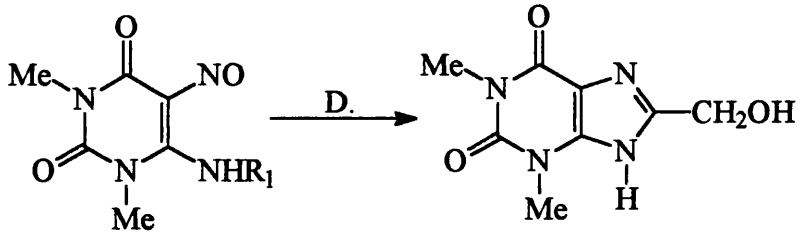
Mechanistically the reaction proceeds through the *insitu* formation of the nitrosonium ion by acid hydrolysis of isoamyl nitrite moiety.

This subsequently leads to the attack of this weak electrophile by the C-5 carbon atom which following resonance stabilisation of the pyrimidine moiety by the electron attracting 3-N-Me group leads to the highly unstable nitroso product as outlined in Scheme 4.5.



Scheme 4.5 The mechanism of nitrosation

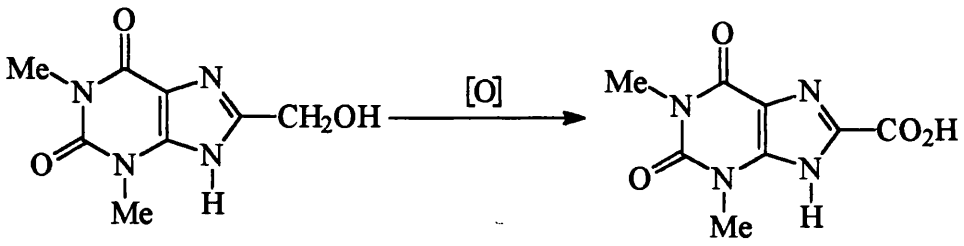
Cyclisation to 8-hydroxymethyltheophylline (15) was subsequently achieved through the cyclodehydration of the C-5 nitroso group and the C-4 alkylamino group via the loss of water in refluxing butanol, Scheme 4.6. The reaction occurs with ease following the decolourisation of the initial nitroso product to afford the ring closed moiety in 54% yield.



(19) (15)

Scheme 4.6 D. BuOH/reflux

Following cyclisation of the nitroso to the ring closed 8-hydroxy compound (15), the required 8-carboxytheophylline (20) was attained through the oxidation of the hydroxy group with potassium permanganate solution to afford (20) in 70% yield *via* Scheme 4.7.



(15) (20)

Scheme 4.7

Analysis of carboxy product via proton NMR proved to be troublesome with a spectrum only achieved in the presence of deuterated trifluoroacetic acid. The loss of the methylenic hydroxy group at δ 4.69 was indicative of the oxidation of the hydroxy moiety. This was further confirmed by analysis of the mass spectra of the carboxy product and the starting hydroxy compound, which on comparison yielded molecular ions (M^+) at 210 and 224 respectively.

However, although this carboxy (20) compound was attained in good yield as a final target compound through the oxidation step, problems were encountered with the solubility of this derivative in organic solvents. Subsequently, the lack of solubility of this carboxy moiety inferred that utilisation of this precursor in the preparation of fluorescent theophylline derivatives was not possible. Subsequently 8-hydroxy derivatives possessing longer alkyl substituents were sought which could be oxidised to a carboxy functionality providing not only a suitable linkage group but also improve the solubility of the carboxy derivative.

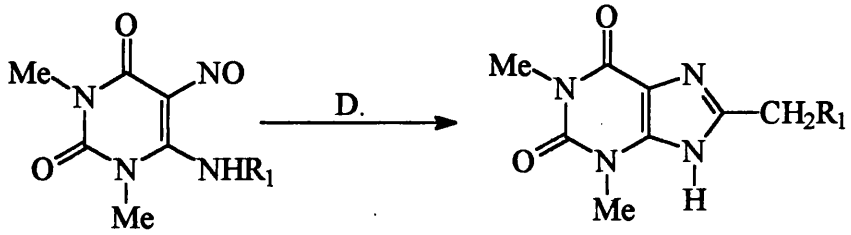
4.3.1. The synthesis of 8-(2-hydroxyethyl)theophylline

As described in the previous section (4.3) the utilisation of the carboxy group for the linkage of amino groups in proteins and peptides in an important process in synthetic and protein chemistry.

Several groups have reported the synthesis of 8-alkylcarboxy derivatives of theophylline in the synthesis of protein conjugates for utilisation as immunogens in the preparation of monoclonal antibodies. Li *et al.*, (1981a) prepared 8-(3-carboxypropyl) theophylline while Oh *et al.*, (1991) prepared the butyl derivative, 8-(4-carboxybutyl) theophylline for utilisation as a possible immunogen. Thus with a view to preparing carboxy derivatives of theophylline possessing such an increase in alkyl chain length, the derivative 8-hydroxyethytheophylline (23) was synthesised.

Preparation of 8-(2-hydroxyethyl)theophylline followed the multi-step procedure of Goldner *et al.*, (1966) from the starting precursor 6-chloropyrimidine (17). Initial preparation of 6-hydroxypropylaminopyrimidine (21) was through reaction of 3-aminopropanol with 6-chloro-1,3-dimethylpyrimidine (17) in 80% yield, Scheme 4.3. Reaction conditions were similar to the synthesis of 6-hydroxypropylaminopyrimidine with the exception of using butanol as the refluxing solvent.

After purification to remove any remaining traces of butanol, the derivative (21) was nitrosated in the presence of isoamyl nitrite to afford (22) in 60% yield with subsequent refluxing in butanol affording the cyclised 8-(2-hydroxyethyl) derivative (23) in 50% yield, Scheme 4.8.



(22) (23)

Scheme 4.8. D. Butanol, reflux $R_1 = CH_2OH$

Attempts to oxidise the hydroxy derivative (23) were not pursued due to a change in approach of the synthetic procedure pursued and the need for hydrophilic linkers for attachment to such derivatives, see section 4.4.

4.3.2 8-methoxyethyl and 9-methoxyethyl derivatives of theophylline

As discussed in section 4.2 and 4.3 synthesis of 8-substituted derivatives of theophylline is possible in good yields particularly in the formation of 8-hydroxyalkyl derivatives of theophylline. Although synthesis of these derivatives was an important goal in the preparation of theophylline targets particularly derivatives possessing functional moieties, it was also important to demonstrate the versatility of this route by directing the synthesis towards 9-substituted derivatives.

Through the work of Bredereck and Föhlisch, (1962) 9-substituted derivatives of theophylline were reported *via* the reduction and subsequent formylation/cyclisation of the starting 5-nitroso precursor. Conditions employed for reduction usually favoured use of electron-transfer reagents such as sodium dithionite or use of zinc in formic acid (Kramer *et al.*, 1977).

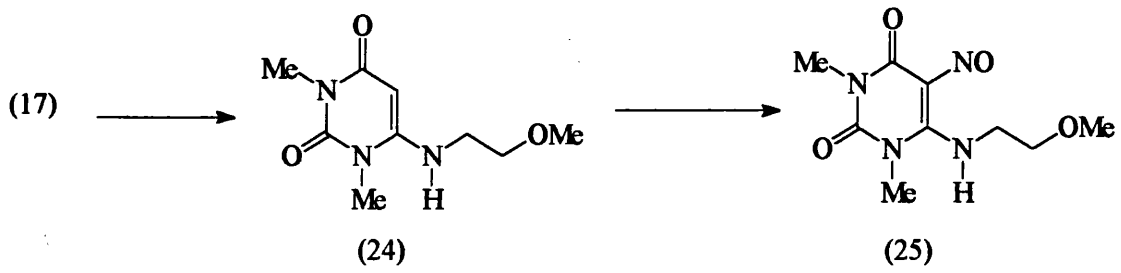
The importance of these nitrosated derivatives lie in their versatility, with ring closure to 8-substituted derivatives through refluxing in butanol while reduction of the nitroso moiety leads to the preparation of useful amine intermediaries. Ring closure is normally effected in refluxing base to afford the 9-substituted derivative.

Initial approaches to the synthesis of the model nitroso derivative (25) was through reaction of 6-(2-methoxyethylamino)pyrimidine (24) with isoamyl nitrite (Scheme 4.9).

Prior to synthesis of nitroso intermediate (25), 6-chloro-1,3-dimethylpyrimidine (17) was substituted with 2-methoxyethylamine to afford (24), as a model derivative, in 80% yield with Na_2CO_3 in refluxing ethanol, Scheme 4.9.

Isolation of this pure compound (24) was by recrystallisation with tetrahydrofuran with characterisation of product by proton NMR confirming the presence of the methoxy singlet at δ 3.08.

This model compound (24) was subsequently nitrosated by dropwise addition of a solution of sodium nitrite in acetic acid following the method of Kramer *et al.*, (1977). Nitrosation proceeded swiftly with reaction reaching completion within 3 h and resulting in a colour change of the solution from clear to purple. Isolation of the nitroso product (25) was by evaporation of the reaction mixture with recrystallisation in ethanol affording a purple crystalline solid in 60% yield, Scheme 4.10.



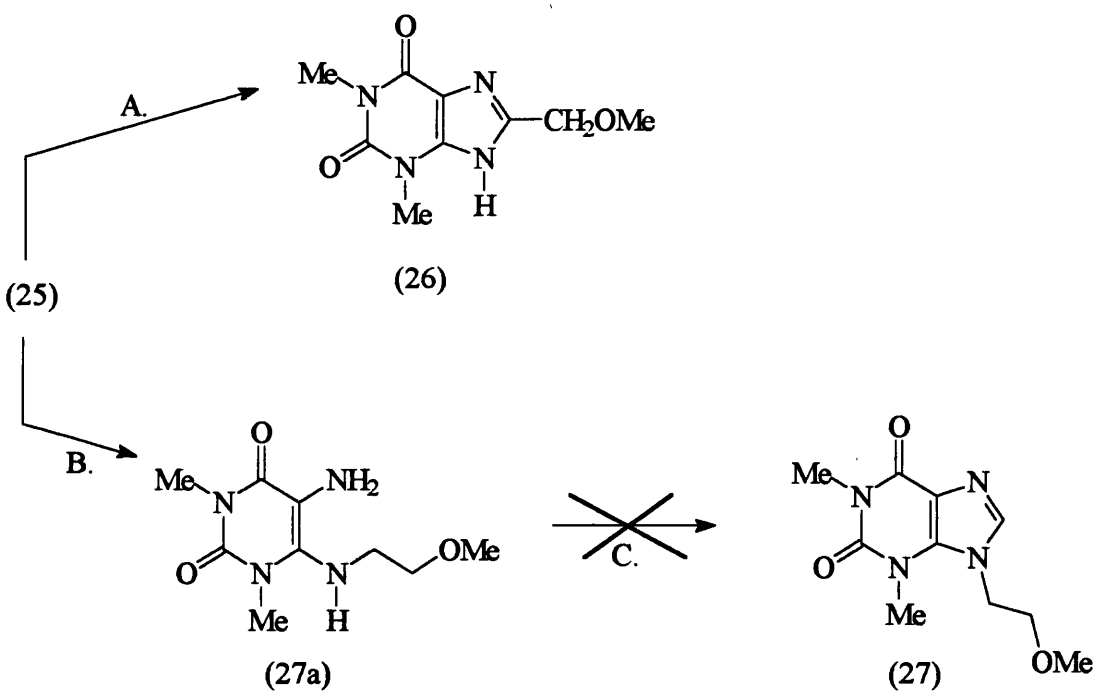
Scheme 4.9

Following this nitrosation, the nitroso compound (25) was used in the synthesis of both 8-methoxymethyl-1,3-dimethylpyrimidine (26) and 9-(2-methoxyethyl)-1,3-dimethylpyrimidine (27).

Thus, thermal cyclisation of nitroso product (25) in butanol afforded (26) as a creamy solid in 50% yield following recrystallisation in ethanol (Scheme 4.10).

Cyclisation proceeded with ease and was followed by the decolourisation of the purple nitroso product in solution.

The synthesis of the 9-substituted product involved the reduction of the nitroso to an amino group in a mixture of zinc/formic acid which was followed by acylation in formic acid. The formamido intermediate formed *insitu* was not isolated but immediately ring closed through base catalysed cyclisation in aqueous NaOH to afford the 9-substituted derivative, (Scheme 4.10).



A. Butanol, reflux; B. zinc/formic acid; C. NaOH (aq)

Scheme 4.10. The synthesis of 8 and 9-derivatives of theophylline using the 5-nitroso precursor

Alternatively, the reduction of (25) was also attempted with aqueous sodium dithionite in ammonia to afford the 5-amino compound (27a) in 50% yield, although this was not confirmed by NMR. Due to the instability of (27a), the amino product

was formylated and cyclised without further purification. Formylation was achieved through reaction with formic acid which following cyclisation of this intermediate with aqueous base yielded (26) as a crude solid. Analysis of the crude product (26) by TLC (10 CH_2Cl_2 : 1 MeOH) inferred the presence of a mixture of products.

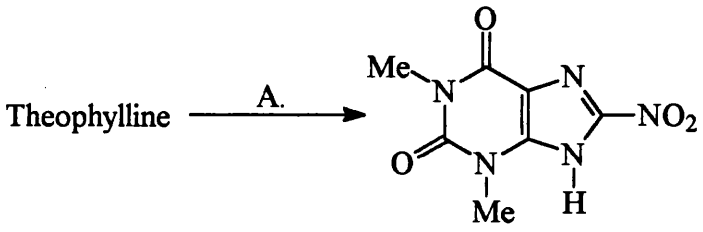
Further analysis by NMR confirmed this observation and the belief that the reaction had not reached completion due to presence of CHO singlet peak at δ 10.13. Isolation of this intermediate was not pursued with attempts to obtain the ring closed 9-methoxyethyl derivative in refluxing base proving to be unsuccessful. Alternative routes were pursued.

4.3.3. 8-nitrotheophylline

Subsequent extension of the modified Traube route led to the synthesis of 8-nitrotheophylline with the possibility of attack by electrophiles (Mosselhi and Pfleiderer, 1993) leading to substitution at the 8-position. The composite electronic system together with the insertion of N-Me groups, electron-releasing groups within the theophylline ring system leads to activation of the 8-carbon leading to attack by electrophiles.

Attempts to synthesise 8-nitrotheophylline by direct nitration of theophyllline proved to be highly successful, through the method of Duesel *et al.*, 1954, Scheme 4.11 Initial conditions involved the reaction of theophylline with nitric acid in the presence of glacial acetic.

The temperature during the nitration step was carefully controlled and did not exceed 90°C, with isolation of the nitrated product as a yellow solid (28) in 60% yield following several washing with water.



(28)

Scheme 4.11. A. HNO_3, CH_3COOH, 87°C, 1h.

Analysis of the proton spectrum in $(CD_3)_2SO$ revealed the presence of the two N-Me groups of the theophylline moiety which were found to resonate as singlets at δ 3.25 and δ 3.43. This was in no way indicative that formation of 8-nitrotheophylline (28) had been achieved and thus further analysis through melting point determination confirmed formation of (28). Thus melting points agreed with literature values of 281 °C *cf.* with 274°C for theophylline.

Following nitration attempts were made to substitute the 8-nitro group with sulphur by reaction with mercaptoethanol. Direct reaction of 8-nitrotheophylline (28) was attempted with mercaptoethanol, initially at 40°C, then at temperatures above 90°C. However electrophilic displacement of the nitro moiety at this position was not successful and alternative routes were sought.

A possible utilisation of the nitro moiety may involve its reduction to the amino group via the use of sodium dithionite (Jones and Robins, 1960) with subsequent attachment of the fluorescent moiety via a thiourea linkage. Thus, a useful comparison between the analogue described and the fluorescent theophylline congeners possessing linker chains, section 4.4, could have been achieved.

4.4 Linker Studies

The use of linkers or spacers is an important requirement of both protein chemistry and organic synthesis. Haptens as well as protein particles require linkage to a large globular protein in order to achieve an immunological reaction. Spacers or linkers are molecules that provide not only an attachment for the hapten but also for the protein complex. In protein chemistry most linkers are based on:

(i) α,ω-alkanediamines (*e.g.* hexane-1,6-diamine, ethane-1,2-diamine)

(ii) amino acids (*e.g.* glycine, lysine)

(iii) monomers or polymers of ethylene glycol (*e.g.* PEG)

(iv) 6-aminohexanoic acid

(v) diaminodipropylamine (DADPA)

These are linkers usually built from individual molecules no more than 10 atoms in length. The best choices have appropriate coupling functionalities on either end and overall hydrophilic character which is enhanced in the presence of polar constituents such as secondary amines, amide linkages, ether groups or hydroxyls. The most common spacers are represented in Figure 4.3, see below.

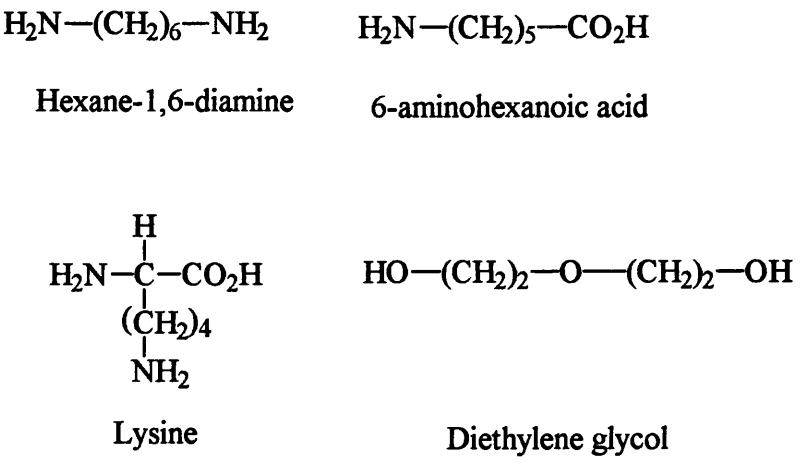


Figure 4.3.

Thus, with a view to incorporating a hydrophilic linker between the theophylline moiety and the fluorophore, a set of linkers were proposed, incorporating either:

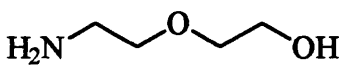
(i) an alkylethoxy group based on the starting precursor 2-aminoethoxyethanol with proposed chain elongation at the hydroxy terminus
(ii) mono-protected polyetherdiamines via selective protection of 3,6-dioxaoctane-1,8-diamine
(iii) α,ω-alkanediamines directly in the reaction with 6-chloro-1,3-dimethypyrimidine (17).

Linkers incorporating ethers were chosen for their increased hydrophilic characteristics.

4.4.1 Linkers based on alkylethoxydiamines

4.4.1.1 The use of 2-aminoethoxyethanol as starting precursor

The synthesis of alkylethoxydiamines and their selective protection were important targets in linker production. Initial work was focused on the use of the starting precursor 2-aminoethoxyethanol as a route to the final α,ω-diaminopolyether chain (Figure 4.4). Elongation was proposed at the hydroxy functionality *via* a Michael addition reaction with acrylonitrile, while protection of the terminal amino group was achieved through use of di-tert-butyl dicarbonate (t-boc) and phenylmethyl chloroformate (Cbz).

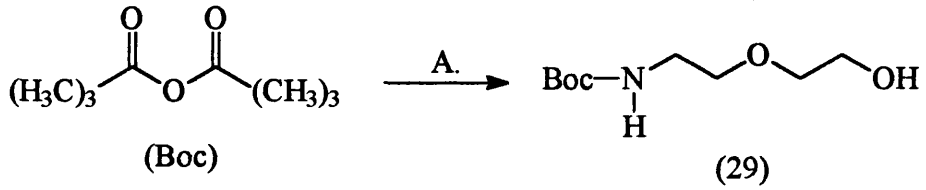


2-(2-aminoethoxy)ethanc

Figure 4.4.

Thus protection of 2-(2-aminoethoxy)ethanol precursor was initially through use of t-boc under standard conditions in the presence of base.

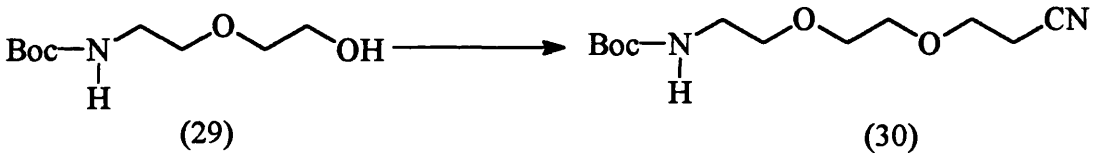
Coupling to the amino functionality was quantitative and resulted in the formation of the carbamate (29) as a clear oil in 99% yield, Scheme 4.12. Analysis of this oil (28) by proton NMR confirmed the presence of the trimethyl of the boc group as a singlet at δ 1.45.



(Boc) (29)

Scheme 4.12 A. $H_2NCH_2CH_2OCH_2CH_2OH$, $(Et_3)N$, RT

Elongation of this ethoxy precursor (29) was achieved by cyanoethylation of the terminal hydroxy group with use of acrylonitrile under basic conditions. Reaction was a standard Michael addition and resulted in the formation of the cyano product (30) in 60% yield following Scheme 4.13. Analysis of the reaction product by I.R. indicated the presence of the sharp nitrile peak at 2200cm^{-1} while analysis of the proton NMR spectrum was indicative of the loss of the methylhydroxy peak at δ 3.75.



(29) (30)

Scheme 4.13

The nitrile was initially synthesised for its versatility and anticipated ease of intergroup conversion in particular the reduction of the cyano group to an amino functionality through route (i) or its subsequent hydrolysis to a carboxylic acid through route (ii), Figure 4.5.

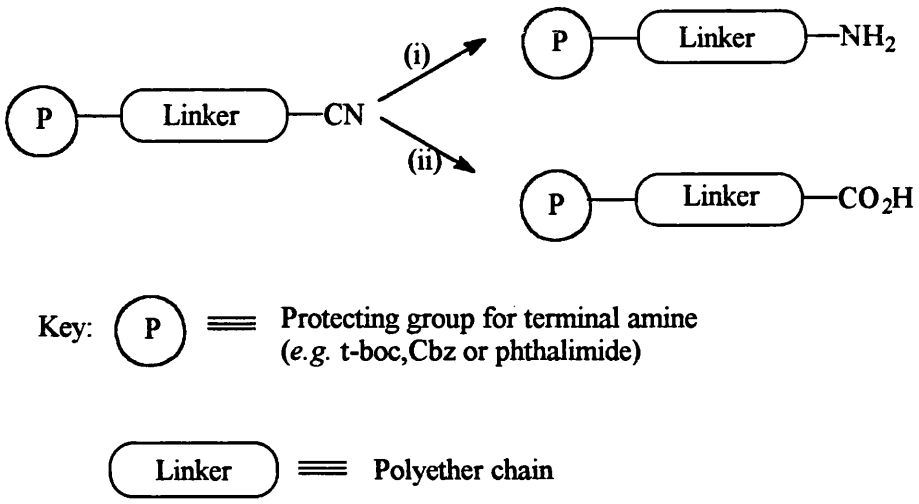
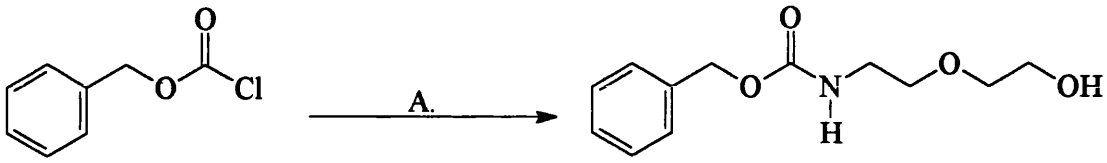


Figure 4.5. Proposed utilisation of 2-aminoethoxy ethanol in linker studies

However attempts to reduce this cyano group (30) met with little success. Catalytic hydrogenation in the presence of palladium on carbon at ambient temperature resulted in the isolation of the starting nitrile. Subsequent hydrogenation under more vigorous conditions, under increasing pressure (100 psi), failed to the reduce this nitrile functionality; thus alternative reducing agents were sought.

One such reducing agent was borane-THF (BH_3-THF complex) which due to its Lewis acidic characteristics cannot be used in the presence of the acid-labile Boc groups. Thus an alternative amino protecting group was sought and benzyloxycarbonyl (Cbz), was chosen not only for its superior acid stability but its resistance to cleavage by reducing agents such as the boron complex.

Thus under similar conditions to the formation of the Boc-protected amine, 2-(2-aminoethoxy)ethanol was protected through the nucleophilic substitution reaction of the amino group with the labile chloro via Scheme 4.14.



(31)

A. $H_2NCH_2CH_2OCH_2CH_2OH$, $(C_2H_5)_3$, CH_2Cl_2, RT

Scheme 4.14.

The carbamate (31) was obtained in 60% yield after isolation of the reaction mixture by washing the organic solution several times with water ensuring removal of both excess starting amino-alcohol and resulting triethylamine hydrochloride from the reaction mixture.

Again, treating this protected carbamate (31) with acrylonitrile gave the Michael addition product (32) as a brown oil in 98% yield, Scheme 4.15. Analysis by I.R. indicated the presence of the sharp nitrile peak at 2250 cm^{-1} while the proton spectrum showed the appearance of the CH_2CN signal at δ 3.01.

(31) (32)

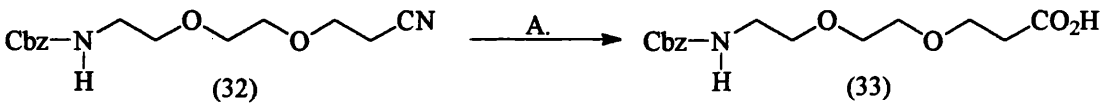
Scheme 4.15.

Reduction of this derivative with BH_3-THF under anhydrous conditions again proved to be difficult with analysis of reaction solution by TLC (silica gel 20 CH_2Cl_2 : 1 MeOH) indicating the presence of a number of products. This was further complicated by the proton NMR spectrum obtained, with assignment of protons proving to be troublesome. Subsequently the isolation of the amino product from this complicated mixture was not attempted as it was believed that partial reduction of the nitrile had occurred leading to difficulties in isolation of target amino compound.

An alternative approach to the reduction of this cyano group was its subsequent hydrolysis to a carboxylic acid which could be functionalised through further modification. Conditions generally favoured were hydrolysis under basic conditions or formation of the methyl ester using a mixture of HCl in methanol with subsequent hydrolysis to the carboxylic acid.

Initial attempts to hydrolyse the cyano moiety (32) in the presence of the Cbz group was met with partial success. The first method adopted was the procedure of Hartley (1962), who oxidised dinitriles of dioxolan to their corresponding amides using hydrogen peroxide which following hydrolysis in base afforded the di-acid.

Subsequently, adaptation of this route involved the use of 30% aqueous potassium hydroxide in hydrogen peroxide under gentle reflux, Scheme 4.16.



Scheme 4.16 A. H_2O_2 in 30% KOH, N_2, reflux, 7 h.

Refluxing of the cyano compound (32) in this mixture was lengthy with reaction completion after 7 h. Following quenching of this hot solution with concentrated hydrochloric acid, the resulting carboxamide was extracted portionwise with diethylether. However, evaporation of the organic portion led to the isolation of a brown gum (33) which could not be triturated even with the most polar of solvents including water and methanol. Subsequent analysis of this gum by proton NMR showed a mixture of products to be present within this reaction mixture. Thus, it was concluded that partial hydrolysis of the cyano moiety to the carboxamide may have occurred together with loss of the protecting Cbz moiety, see Figure 4.6 below. Although this is only a hypothesis the difficulty in re-isolation of the starting nitrile suggested hydrolysis to this carboxy moiety.

Figure 4.6

Further attempts to hydrolyse this nitrile moiety (32) in refluxing base were also met with disappointment with the nitrile moiety proving to be particularly stubborn to base hydrolysis. Refluxing in dry HCl/MeOH also proved to be disappointing as methylester production was not seen with recovery of starting nitrile (32). The lack of success in utilisation of the nitrile moiety proved frustrating with a mixture of products seen during reduction or recovery of starting material upon hydrolysis.

Thus it was concluded that the presence of the two ether functions may somehow be causing deactivation of the nitrile group leading to resistance to intergroup conversion. Although this may be somewhat surprising particularly in view of the work of Newkombe *et al.*, (1991) who were able to hydrolyse alkyethoxy nitriles to their corresponding methylesters by refluxing in dry HCl/MeOH, in the Pinner reaction, with hydrolysis affording the carboxylic acid.

However, in their approach the starting cyano precursors possessed only one ethoxy moiety which may have less of a deactivating effect than in the case of cyano compound (32) where two are present. Thus further work on the utilisation of ethoxy nitriles needs to be pursued.

4.4.1.2 The use of 3,6-dioxaoctane-1,8-diamine as starting precursor

As outlined in the previous section (4.4.1.1) attempts to functionalise amino protected diethoxy derivatives of nitriles (30) and (32) proved to be difficult and met with little success. Therefore, attempts were made to selectively mono-protect the commercially available 3,6-dioxaoctane-1,8-diamine (Figure 4.7) starting precursor in the presence of phenylmethyl chloroformate (Cbz).

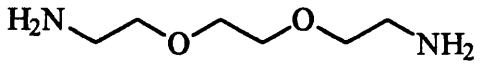
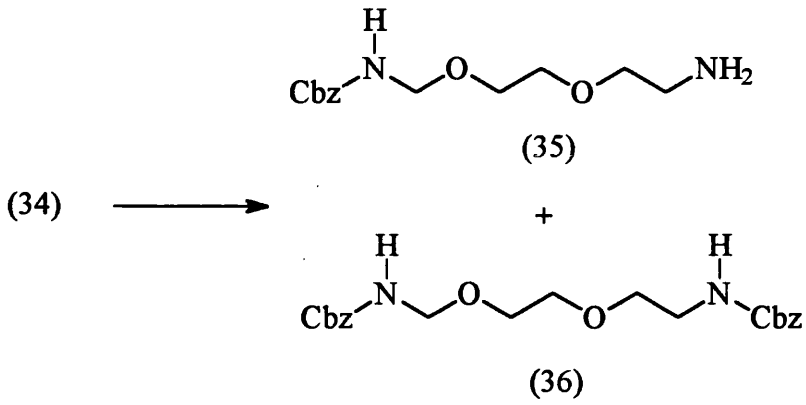


Figure 4 7.

The selective protection of α,ω-alkanediamines has been associated with Atwell and Denny (1984) who synthesised mono-protected intermediates of poly diamines in the presence of the Cbz group.□ Attempts to locate procedures to selectively protect diamino polyethers proved to be difficult, therefore the use of Cbz was an attractive option in this synthetic approach.

In the initial reaction, it was critical that an excess of the starting 3,6-dioxaoctane-1,8-diamine (34) was in solution prior to addition of the amino protecting group. The rationale was that the large excess of the starting precursor would discourage the formation of the bis-Cbz product and allow selective protection of one free amino group.

Thus a two molar excess of the precursor (34) was allowed to react by dropwise addition with phenylmethyl chloroformate in the presence of base, Scheme 4.17.



Scheme 4.17.

To achieve complete reaction between the diamine and the Cbz protecting group, the reaction mixture was stirred overnight at room temperature. After completion, the solution was washed with a small quantity of water ensuring removal of excess starting diamine without loss of the highly soluble mono-Cbz protected product (35).

Analysis of the reaction mixture by TLC proved to be disappointing as two spots were located, of which the lower Rf was attributed to the mono-Cbz derivative (35), which was also ninhydrin active, while the one with higher Rf was assigned to the bis-Cbz protected compound (35). Further analysis by proton NMR indicated that the major product of the synthesis was the bis protected derivative, therefore, attempts were made to isolate these products by extraction of the final reaction mixture.

Isolation of the mono-Cbz protected compound (35) was achieved by washing the organic layer with 2M HCl, extracting the aqueous portion then washing with 2M NaOH with subsequent extraction of this basic layer with ethyl acetate. Evaporation of solvent afforded the mono-protected amine (35) in 20% yield as an oil.

While the bis-protected by-product (36) was isolated by evaporation of the organic portion to afford an oil which crystallised on standing in 40% yield.

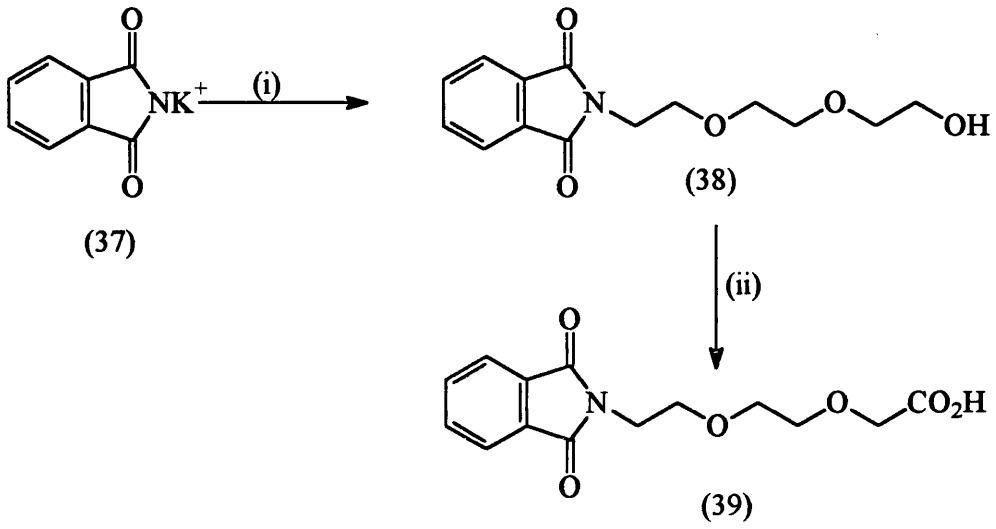
Repeating this procedure with larger molar excesses of the polyether diamine in the reaction mixture had little or no improvement in the yields obtained for the mono protected product. This approach was thus abandoned due to the difficulty in obtaining high yields of the mono-protected derivative.

4.4.1.3 The use of 2-[2-(2-chloroethoxy)ethoxy]ethanol

The final approach in the use of ethoxy moieties led to use of 2-(2-(2-chloroethoxy)ethoxy)ethanol by reaction with potassium phthalimide, Scheme 4.18. The reaction is based on the Gabriel procedure which has been modified by Maeda *et al.*, (1982) and Botros and co-workers (1986) in the preparation of amino-protected diether derivatives.

The procedure followed reaction of 2-(2-(2-chloroethoxy)ethoxy)ethanol starting precursor with potassium phthalimide (37) in DMF (Scheme 4.18). Isolation of the phthalimido product (38) was by flash column chromatography in 20-35% yield maximum. Increasing reaction temperatures had little or no effect on the yield of the final product and subsequently, this derivative was used without further purification in the proceeding step.

Thus before removal of the phthalimide group to afford the amino compound, the hydroxy terminus was oxidised to the carboxy derivative in the presence of potassium chromate and sulphuric acid in acetone, Scheme 4.18.

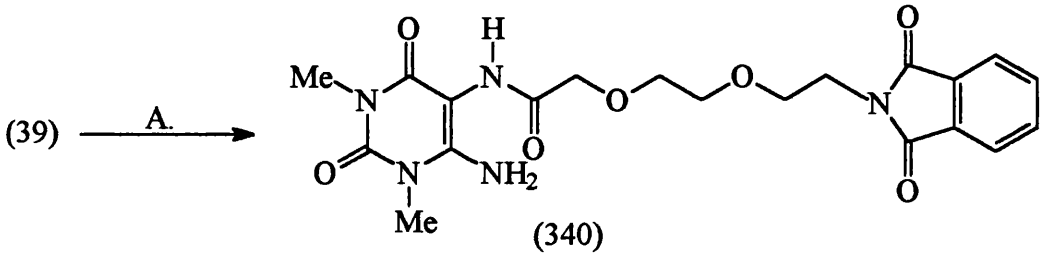


(37) (38) (39)

(i).$Cl(CH_2)_2O(CH_2)_2O(CH_2)_2OH$, DMF,110°C;
(ii) K_2CrO_3/H_2SO_4, acetone, RT, 4 h.

Scheme 4.18.

The carboxy derivative (39) was obtained in 60% yield with no isolation of the aldehyde intermediate, as confirmed by the mass spectrum of the carboxy moiety with a molecular ion of 294. Subsequently an attempt was made to utilise this carboxy derivative, prior to removal of the phthalimide group, through reaction with 5,6-diamino-1,3-dimethylpyrimidine (14) in the presence of dicyclocarbodiimide in (Shimada *et al.*, 1991), Scheme 4.19.



(340)

Scheme 4.19 A. (1), DCC, DMF

This route was primarily adopted as a means of obtaining the 5-amido intermediate (40) through condensation of the acid (39) with 5,6-diaminopyrimidine (14).

In section 4.3 attempts were made to obtain 8-hydroxymethyltheophylline (15) via the use of the diaminopyrimidine precursor (14). Yields were low with isolation of the final cyclised product (15) proving to be difficult. Thus, since polyether chains were required as hydrophilic linkers and with attempts at obtaining mono-protected aminoethoxycarboxy derivatives (see section 4.4.1) proving to be difficult, it was decided that the phthalimido derivative (39) was a useful starting precursor.

Through Scheme 4.20 the formamido product (40) was obtained as a crude solid. Analysis by TLC indicated the presence of a number side products present within the reaction mixture. Of these the phthalimido product (40), possibly the cyclised product and theophylline were assigned. Subsequent purification of the reaction mixture by column chromatography led to the isolation of the phthalimido product as a yellow solid in 12% yield.

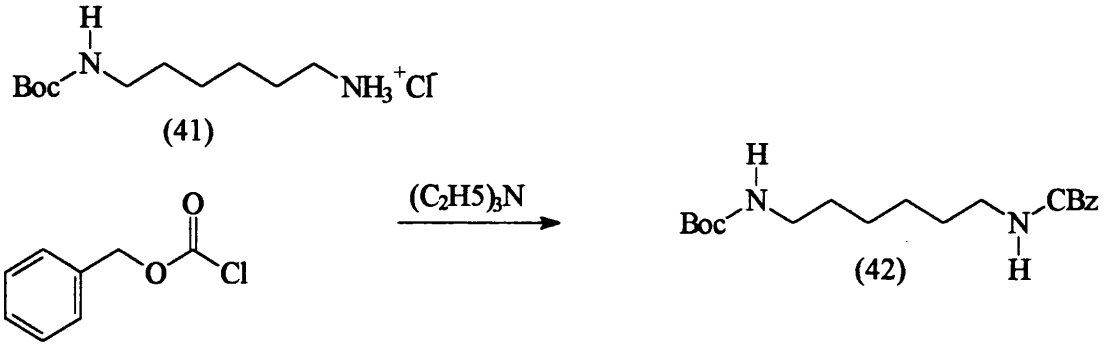
Through analysis of the ^1H proton spectrum the phthalimide ring system was found to resonate at δ 7.86 while mass spectrum confirmed the isolation of the amido intermediate (40) with (M^+) 445. Attempts to cyclise this amido intermediate (40) in aqueous base were not pursued due in effect to the low yield of this procedure and the formation of undesirable side products.

However, utilisation of this method requires a change in reaction solvent since dimethylformamide has been reported as a cyclisation agent for 5,6-diaminoprecursors in the Traube route (Speer and Raymond, 1953). Also, the starting diaminopyrimidine is highly unstable and requires purification prior to use. Thus in view of these problems, an improvement in the yield of the final product could be achieved by prior purification of the diamino compound.

4.5. Utilisation of α,ω-alkanediamines

4.5.1 The use of N'-Bochexane-1,6-diamine as starting precursor

Commercially available N'-bochexane-1,6-diamine (41) was protected with phenylmethyl chloroformate (Cbz) under basic conditions using standard procedures. The reaction proceeded over a 24 h period and resulted in a product protected at both terminal amino ends, with a Boc group at one end and Cbz at the other, scheme 4.20.

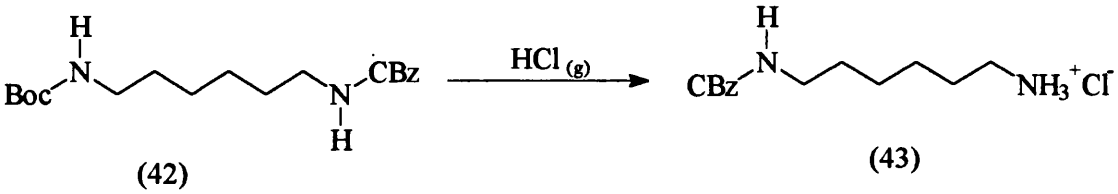


Bis protected amino hexyl compound

Scheme 4.20

The bis-protected compound (42) was isolated in 86% yield following successive washing of the product in water and then acid.

Removal of the Boc amino protecting group from the bis-protected product (42) was achieved using HCl gas in dichloromethane. Deprotection was achieved without loss of the Cbz moiety in yields of 95% and above to afford the mono-Cbz protected hydrochloride (43) as a solid, Scheme 4.21.

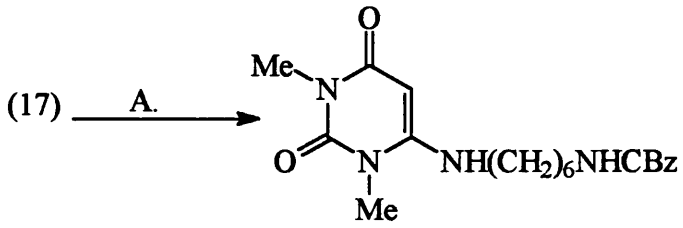


(42) (43)

Scheme 4.21

This method is a standard procedure for the removal Boc groups from amino functionality and is particularly useful method for the selective removal of one amino protecting group in the presence of another. Since the Cbz moiety is acid stable, Boc groups can be selectively removed from the terminal amino group of the hexanediamine while still protected at the other. The resulting hydrochloride salt (43) is thus used without further purification in the reaction with 6-chloropyrimidine (17).

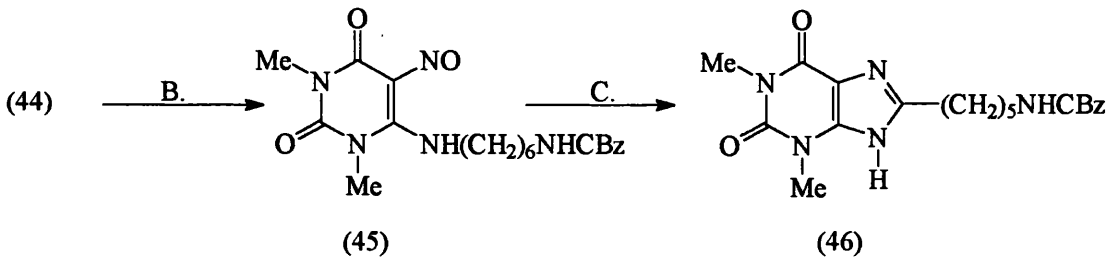
Subsequent reaction of the mono-Cbz hydrochloride (43) with 6-chloropyrimidine derivative (17), in refluxing 1,4-dioxan (Scheme 4.22) afforded the 6-substituted-Cbz protected amino compound (44) in 60% yield following recrystallisation in ethanol.



(44)

Scheme 4.22 A. (42), Na_2CO_3, 1,4-dioxan, reflux

Nitrosation of the 6-substituted Cbz-protected product (44) was achieved in the presence of isoamyl nitrite in aqueous HCl *via* the reaction outlined in Scheme 4.23. Isolation of this nitroso derivative (45) was in 73% yield with subsequent cyclisation in butanol affording the cyclised derivative (46) in 97% yield. Scheme 4.25. Purification by recrystallisation in butanol afforded (46) in analytically pure form for use in the deprotection step.



Scheme 4.23 B. isoamyl nitrite, HCl, R.T., C. butanol, reflux

Deprotection of the amino protecting group was achieved using hydrogen bromide in acetic acid (Ben-Ishai, 1953). Isolation of the hydrobromide salt (46) was attained in 99% yield after trituration with diethyl ether. Analysis of the proton NMR spectrum confirming the presence of the broad singlet $NH^{+}_{3}Br$ peak at δ 9.00 with the disappearance of the Cbz phenyl peak at δ 7.01.

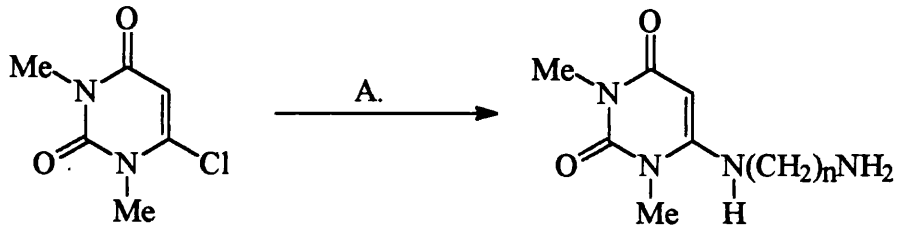
Thus the successful synthesis of 8-(5-Aminopropyl)-2,4-dimethylpurine-1,3-dione (46) as the hydrobromide salt, through this multi-step procedure has led to the utilisation of alkanediamines as linkers in the final fluorescent theophylline moiety.

4.5.2 The synthesis of 8-(ω-aminoalkyl)theophyllines

The initial attempts to synthesise appropriate theophylline derivatives for attachment of a fluorescent electrophiles were met with partial success. The synthesis of 8-hydroxymethyl and 8-hydroxyethyltheophylline were attained in high yields, 54% and 82% respectively, from the starting 6-chloropyrimidine (17). However, subsequent oxidation of 8-hydroxymethyltheophylline to the 8-carboxy derivative resulted in a product which was insoluble in most polar solvents (*e.g.* methanol, DMF) apart from trifluoroacetic acid.

Therefore, due to the insolubility of such carboxy derivatives other synthetic targets were sought containing appropriate linkers and a terminal fuctionality for the attachment of the fluorescent probe.

Subsequent investigation has therefore led to the development of a series of 8-(ω-aminoalkyl)theophyllines as possible targets for the attachment of fluorescent electrophiles. Optimisation of chain length was achieved by use of appropriate starting α,ω-alkyldiamines through the procedure of Fuschs *et al.*, (1978) utilising the 6-chloropyrimidine derivative (4) as a starting precursor, Scheme 4.24.



(17)

(47) n = 6
(48) n = 9
(49) n =10
(50) n = ethoxy

A. alkane-α,ω-diamine, dioxane, Na_2CO_3, reflux, 4 h.

Scheme 4.24

The initial conditions of the substitution reaction involved refluxing starting α,ω-alkyldiamines (n = 6, 9, 10, alkylethoxy) in ethanol in the presence of sodium carbonate.

However analysis of the reaction mixture by TLC was indicative of the formation of three products with the formation of an ethoxy derivative as a side product at Rf 056 compared with required mono product at Rf 0.45. Therefore, in order to eliminate the possibility of such side reactions with ethanol, an inert solvent was desired and thus our solvent of choice was 1,4-dioxan.

The substitution reaction for all spacers was subsequently carried out in boiling 1,4-dioxan containing an excess of the starting diamine and base. The reaction conditions were carefully controlled and an a 2.5 molar excess of the diamine was used as an optimal quantity to achieve the maximum possible yield of the mono-product.

Isolation of the substituted 6-aminoalkylaminopyrimidine derivatives was through removal of excess starting diamine *via* an aqueous wash and removal of solvent. thus ensuring ensured possible interferences in the proceeding benzylation step. Thus analysis of crude amine products by TLC confirmed the presence of two components, the major 6-amino product being ninhydrin active and located at an of Rf 0.15 while the bis product is seen at Rf 0.63., Figure 4.8.

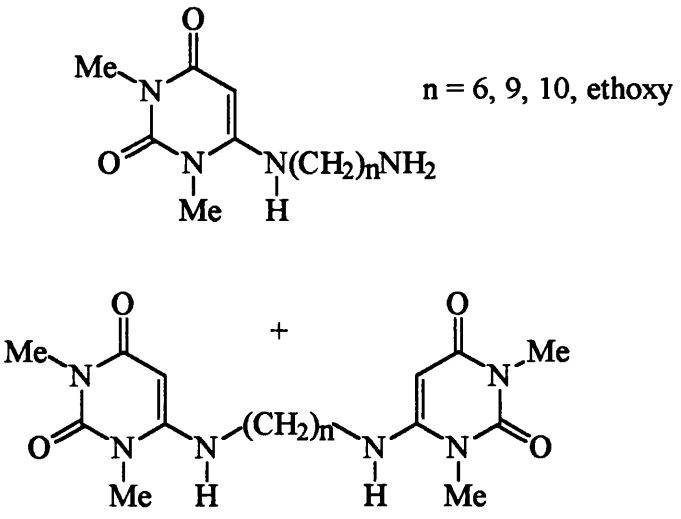


Figure 4.8.

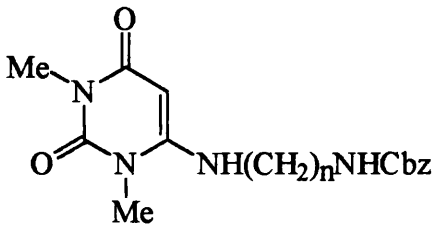
Attempts to isolate this major product, the 6-aminoalkylamino derivative, through recrystallisation and column chromatography proved troublesome. Subsequently, the product was used in the next step without further purification.

However, through our earlier work, isolation of the 6-aminononyl (51) and 6-aminodecyl (52) derivatives was possible through recystallisation in aqueous ethanol in yields of 65% and 64% respectively. However for ease of synthesis and isolation of the Cbz protected product, the 6-amino derivatives were not purified and subsequently were used in the benzylation step fairly swiftly.

Nitrosation as previously recorded for the 6-hydroxyalkylpyrimidines (section 4.2) was achieved directly by use of isoamyl nitrite under mild acid conditions. However, the presence of free amino groups presented possible side reactions occurring, in particular the formation of unwanted nitrosoamines. Subsequently, prior to nitrosation these 6-minoakylamino derivatives were protected as their Cbz counterparts.

A number of amino protection groups were considered, however only phenylmethyl chloroformate (Cbz) matched the criteria required including being stable in acid environments as well as ease of removal without disruption of the theophylline moiety. Thus, the 6-substituted derivatives of the alkylamines (n=6, 8, 9) and alkylethoxyamine ($CH_2CH_2OCH_2CH_2OCH_2CH_2$) were protected through reaction of phenylmethyl chloroformate (Cbz) in the presence of triethylamine, Scheme 4.25.

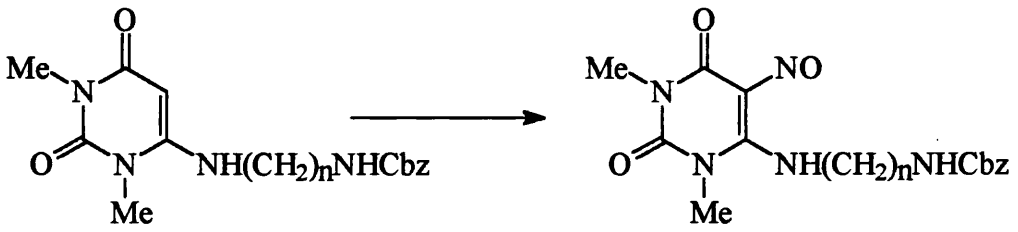
Reaction times were 24 h stirring at room temperature with isolation of crude Cbz product *via* concentration of solvent *in vacuo*. Purification of these protected amino 6-alkylamino derivatives was through column chromatograpy with isolation of these alkyl linkers (n = 6, 9, 10) using the system 4 chloroform : 1 EtOAc. While isolation of the alkylethoxy linker required a more polar system and was achieved in 5 EtOAc : 1 MeOH in 44 % yield. Table 4 represents the yields obtained



n	compound number	% Yield
6	53	54
9	54	43
10	55	54
ethoxy	56	44

Table 4.

Nitrosation of these Cbz-protected amines followed with ease *via* reaction with isoamyl nitrite and resulted in a number nitroso derivatives which were all isolated as oils, Scheme 4.26. Subsequently, these derivatives were used in the cyclisation step without further purification.

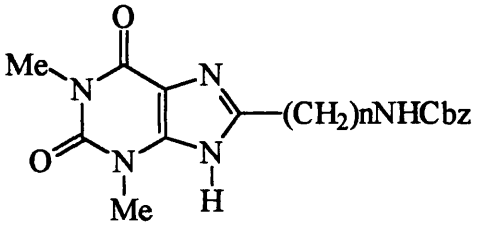


[n = 6, 9, 10, ethoxy]
(53), (54), (55), (56)

(57), (58), (59), (60)

Scheme 4.25

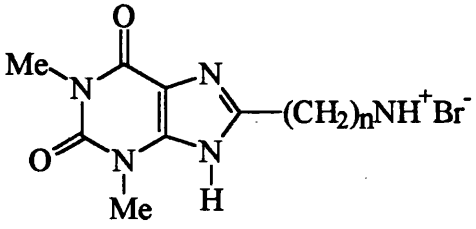
The instability of these nitroso derivatives enabled the cyclisation step to proceed swiftly with the cyclised products attained in good yields, Table 4.1.



n	compound number	% Yield
5	61	97
8	62	37
9	63	45
ethoxy	64	68

Table 4.1

Deprotection of the terminal amino group was achieved through reaction with hydrogen bromide in acetic acid, with trituration in diethyl ether affording the penta (65), octa (66) and nonamethylene (67) derivatives as the hydrobromide salts in high yields. However, isolation of the ethoxy derivative (68) required trituration with ethyl acetate to afford the hydrobromide as a crystalline solid. Yields obtained for the deprotection step is depicted in Table 4.2.



n	compound number	%Yield
5	65	100
8	66	100
9	67	87
ethoxy	68	100

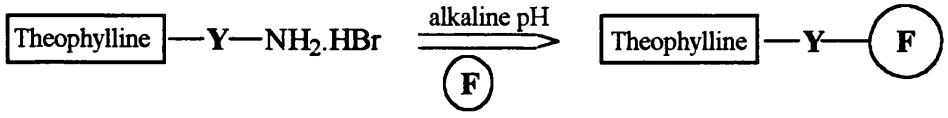
Table 4.2

Analysis of the proton NMR spectrum reveals the presence of the broad 3-H proton peak at δ 7.71 (as indicated for the nonamethylene derivative, see experimental) indicative of the NH_3^+ group of the hydrobromide salt with no protons at the aryl region indicating the loss of the Cbz protecting group. Subsequent conjugation to the FITC congener is outlined in the next section (4.6) *via* reaction with the hydrobromide salts synthesised.

4.6 Synthesis of fluorescent congeners

Fluorescent probes have been utilised in biochemistry either for analysing quantitatively functional groups in proteins or probing the environment around functional groups (Okamato et al, 1982). The most common probes are based on fluorescein (*e.g.* FITC, DTAF, rhodamine) and sulphonyl chlorides, in particular dansyl chloride (5-dimethylamino-1-naphthalenesulphonyl chloride). Utilisation of these probes as fluorescent labels is through reaction with free amino groups in aqueous solutions under alkaline conditions. Yields for coupling are almost quantitative with isolation of products as highly fluorescent derivatives.

To advance the utilisation of these fluorescent probes more widely, theophylline derivatives were coupled to FITC, dansyl chloride and DTAF under controlled basic conditions, Figure 4.9.



Key: F ≡ Fluorescent electrophile

1. FITC
2. Dansyl chloride
3. DTAF

Y = $(CH_2)_5$, $(CH_2)_8$, $(CH_2)_9$, $CH_2OCH_2CH_2OCH_2CH_2$

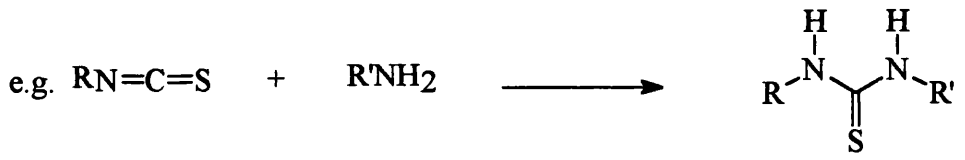
Figure 4.9 A diagram depicting the general synthetic strategy in the development of fluorescent congeners for theophylline

Investigations led to the synthesis of a series of highly fluorescent theophylline congeners, possessing both an alkyl and alkylethoxy linker, which were subsequently used to assess the FCFD assay for theophylline.

Therefore, functionalisation of the fluorescent chromophore onto the theophylline moiety involved reacting the hydrobromide salts of the 8-alkylamino and 8-alkylethoxy derivatives of theophylline with fluorescein isothiocyanate (FITC), dichlotiazinylaminofluorescein (DTAF) and dansyl chloride under controlled basic conditions.

4.6.1 FITC conjugation to primary amines

Fluorescein isothiocyanate (FITC) is an electrophile used primarily in the labelling of proteins and peptides through reaction with free amino groups. Final products are highly fluorescent derivatives which are coupled to the FITC moiety through a thiourea linkage, scheme 4.26.

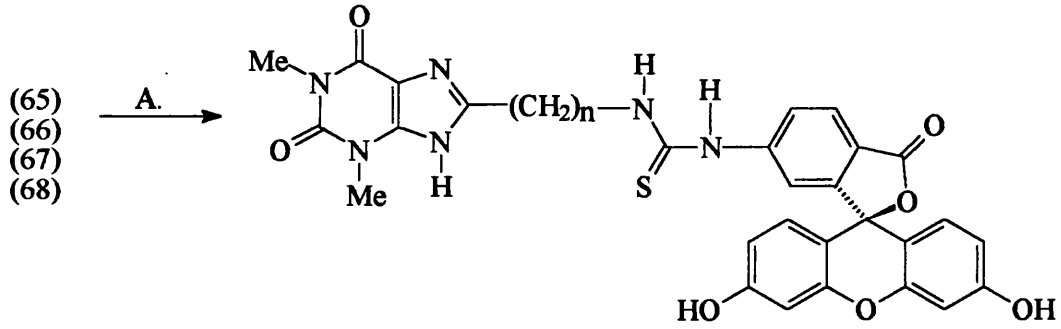


Scheme 4.26

The aim of the synthetic procedure, as discussed in Section 4.2, was to synthesise 8-substituted fluorescent derivatives of theophylline possessing an alkylamino or alkylethoxy linker between theophylline and the fluorophore.

The hydrobromide salts of the 8-alkylaminotheophyllines containing a penta, octa, nona methylene and an ethoxy linker were functionalised onto the fluorescein moiety by careful choice of reaction conditions.

Reactions were performed under controlled basic conditions by reacting the salts of these derivatives with fluoresceinisothiocyanate (isomer I) in a mixture of 1:1 dioxan/1M K_2CO_3 and stabilising the pH at 9.0 $\pm$ 0.1 by further addition of base. The reaction was monitored by TLC for the formation of the highly fluorescent product, Scheme 4.27.



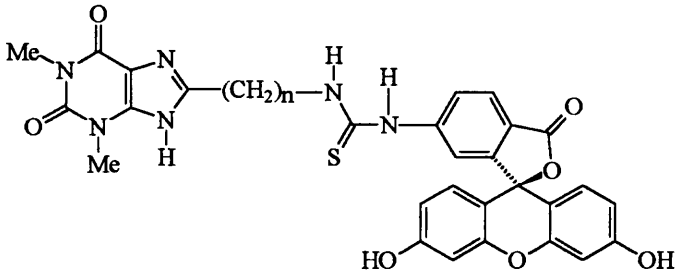
(69)-(72)

A. n=5, 8, 9, pH 9.0$\pm$ 0.1, 1M K_2CO_3, dioxan, RT.

Scheme 4.27

After 3 h stirring at room temperature, the reaction mixture was acidified to pH 6.0. This mixture was then taken up in water, and subsequently freeze-dried for 24 h to afford the crude product as an orange yellow solid.

Analysis of the crude thiourea products by TLC inferred the presence of a number of impurities. Purification through flash column chromatography on silica led to isolation of the desired 8-conjugated products as orange solids in high yields, Table 4.3.



n	compound number	% Yield
5	69	50
8	70	72
9	71	68
ethoxy	72	77

Table 4.3

Analysis of these products were through proton NMR and COSY.

Figures 4.10 and 4.11 show the ^1H NMR and COSY spectra respectively, of the $(CH_2)_9NHCSNH$-linked fluorescein-theophylline in $(CD_3)_2SO$. The spectra clearly show the three parts of the structure- theophylline, linker and fluorescein.

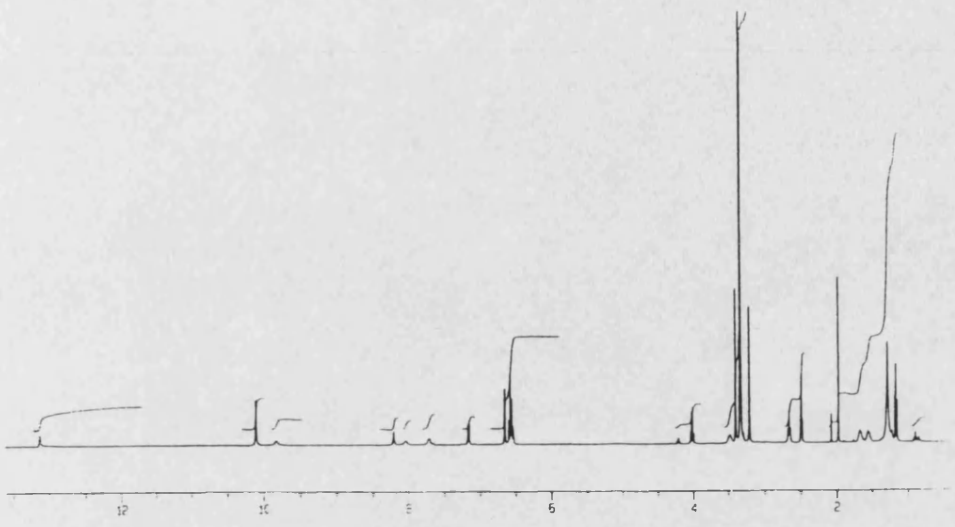
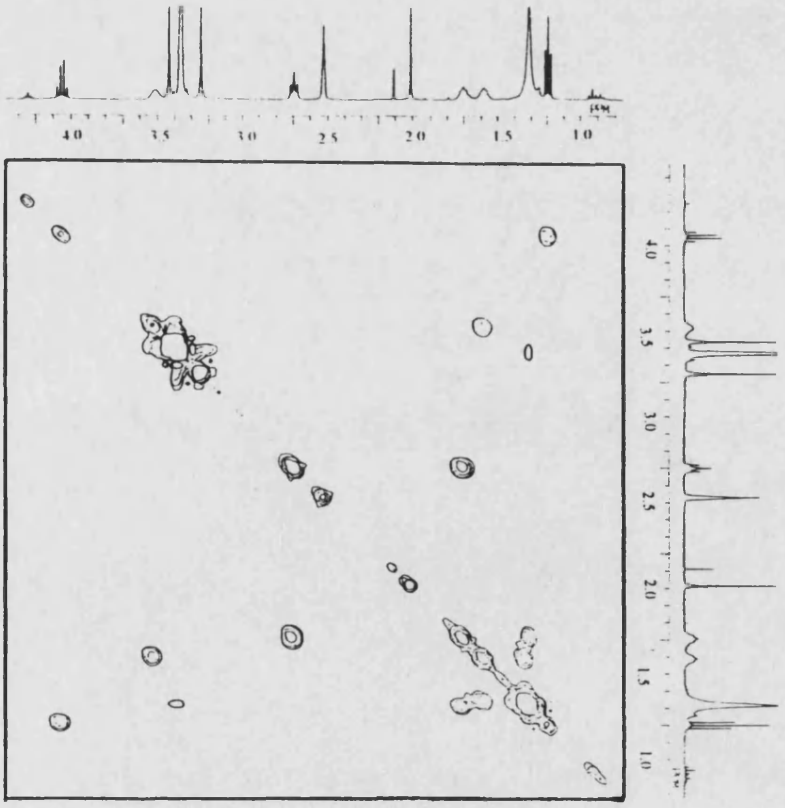


Figure 4.10.

Figure 4.11



The two N-CH_3 groups of the theophylline moiety resonate as singlets at δ 3.22 and δ 3.41, identical to the chemical shifts for the precursor 8-(9-aminononyl)theophylline hydrobromide. The purine 9-NH also resonates at similar chemical shifts in the conjugate and the precursor (δ 13.1 and δ 13.18, respectively).

The COSY spectrum allows assignment of the resonance of the nonamethylene unit of the linker. The central $(CH_2)_5$ unit appears as a broad peak centred around δ 1.29 whereas the other four methylenes appear as discrete signals. Interestingly, the broad quintet at δ 1.56 couples with the broad peak at δ 3.48 and the broad quintet at δ 1.68 couples with the triplet at δ 2.67.

Since the signal at δ 2.67 can be assigned to the purine-CH_2 on the basis of chemical shift (*cf.* δ 2.68 in the precursor) and mutilpicity, then the signal at δ 1.68 can be assigned to the purine-CH_2CH_2. Similarly, the signal at δ 3.48 corresponds to CH_2N, on the basis of chemical shift. Thus the signal at δ 1.56 must arise from CH_2CH_2N. The order of the chemical shifts of purine-CH_2CH_2 and CH_2CH_2N is unexpected, considering simple inductive effects. The thiourea NH signals appear at δ 8.06 (CH_2NH) and δ 9.86 (Ar-NH).

In the fluorescein moiety, the signals from the benzene ring and from the xanthene appear in separate clusters. The protons of the fewer form a typical trisubstituted benzene spin system. H-3 resonates as a doublet at δ 7.17 ortho-coupled to H-4 (δ 7.70) whereas the isolated proton at H-6 appears as a broad singlet at δ 8.22, Figure 4.12.

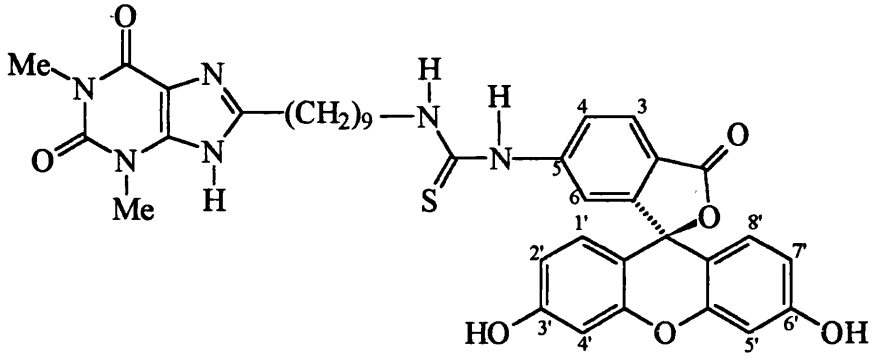


Figure 4.12

The signals for the xanthene show the symmetry of this group. Again, the C-H signals indicate a trisubstituted benzene. H-1' and H-8' form a doublet at δ 6.58 which is ortho coupled to H-2' and H-7' at δ 6.56. H-4' and H-5' appear as a narrow doublet at δ 6.67, which is meta coupled to H-2' and H-7'. The phenolic OH resonate together as a singlet at δ 10.13.

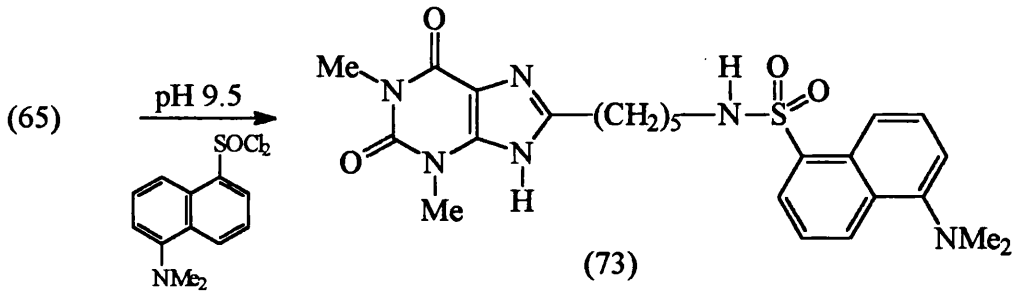
4.6.2 The use of dansyl chloride

Through the successful coupling of the hydrobromide salts to the FITC congener, the pentamethylene analogue was used as a model compound in the reaction with other fluorescent electrophiles.

The conjugation of 8-aminoalkyl and 8-aminoethoxyalkyl derivatives of theophylline to FITC (section 4.6.1) was an important goal in the preparation of fluorescent labels of theophylline. The ease of synthesis as well as isolation of the required congeners in high yields provided a route to the synthesis of other fluorescent derivatives. Thus, subsequent utilisation of the synthetic procedure enabled conjugation of the pentamethylene derivative to dansyl chloride (5-dimethylamino-1-napthalenesulphonyl chloride).

Dansyl chloride was chosen as alternative label due to its successful use as a fluorescent handle for the study of proteins (Gray, 1967). Reactions with primary and secondary amino groups as well as phenolic hydroxyl and imidazole groups of amino acids occur under mildly alkaline solutions to yield sulphonyl derivatives which fluorescence strongly in organic solvents (Airhart *et al.*, 1973).

Conjugation of dansyl chloride to the pentamethylene derivative (65) was achieved through Scheme 4.29. The pH of the reaction was carefully maintained at 9.5, with isolation of the dansyl product (73) in 54% yield as a green solid. A pH of 9.5 was chosen as the optimal pH of coupling as outlined by the work of Gray (1967) who optimised the coupling of amino acids to dansyl chloride between pH 9.5-10.5.



Scheme 4.28

Subsequent analysis of the proton NMR spectrum (Figure 4.13) inferred successful conjugation of dansyl chloride to the theophylline moiety.

Figure 4.13 clearly shows the protons attached to the purine moiety, linker and the dansyl portion. The methylene protons within the linker moiety appear as multiplet signals resonating at δ 1.14, 1.29 and 1.49.

The NHCH_2 group appears to be downfield at δ 2.76 while the purine N-CH_3 groups attached to the theophylline moiety appear as singlets at δ 3.22 and δ 3.39 together with the purine-CH_2 protons.

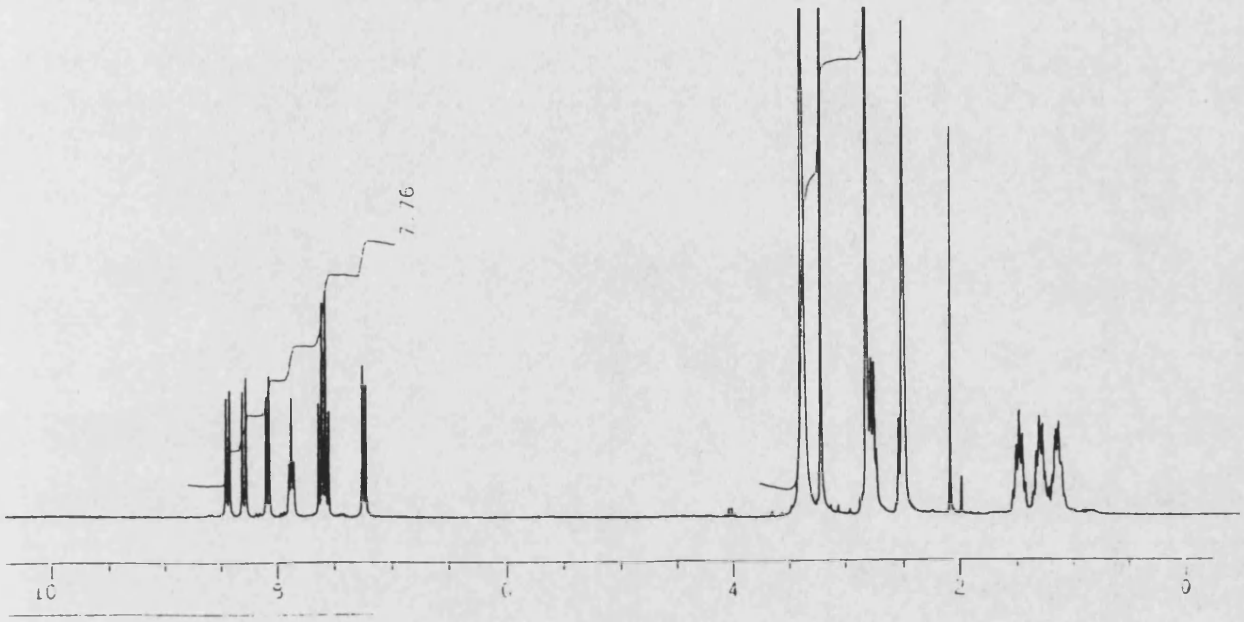
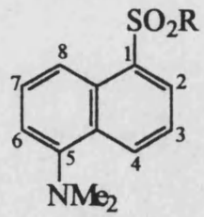


Figure 4.13.

The naphthalene portion is identified by the singlet $N(CH_3)_2$ peak at δ 2.81 while the aromatic signals clustered in the region δ 7.2-8.4 are allocated to the naphthalene ring system of the fluorescent portion, Figure 4.14.



R = pentamethylene derivative of theophylline

Figure 4.14

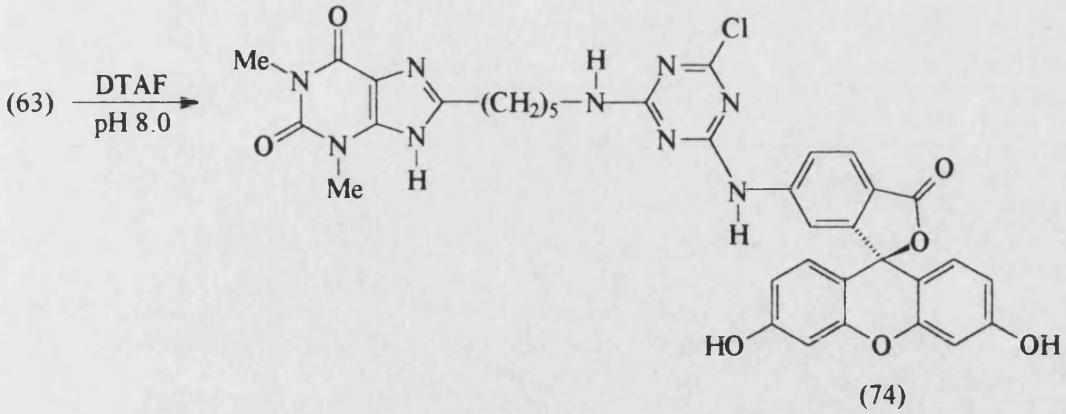
The protons were assigned without the aid of a COSY spectrum therefore all protons available in the naphthalene aromatic region were allocated as Aryl-H and appeared as doublets.

4.6.3 The use of DTAF (Dichlorotriazinylaminofluorescein)

The successful conjugation of both FITC and dansyl chloride to the hydrobromide salts of theophylline precursors led to the extension of this procedure towards coupling with other fluorescent congeners. Thus DTAF, an important derivative of fluorescein, was chosen as a suitable probe for the labelling of theophylline.

DTAF has found extensive use in the labelling of proteins reacting with free amino groups through substitution at the triazine ring resulting in the formation of highly fluorescent cholrotriazinylaminoflurescein (CTAF) derivatives. Thus through the method of Mahoney and Azzi (1987), DTAF was successfully conjugated to the pentamethylene derivative (63) via Scheme 4.30 under controlled basic conditions, pH 8.0.

Isolation of the fluorescent CTAF derivative (74) was through column chromatography in 68% yield as an orange solid. In comparison to the conjugation of both FITC and dansyl chloride to the theophylline derivative possessing a pentamethylene linker, DTAF required less alkaline conditions, pH 8.0 *cf.* pH 9.0 for FITC and pH 9.5 for dansyl chloride. Also pH control was not as stringent as for coupling to FITC although throughout the reaction the pH was maintained at 8.0, Scheme 4.30.



(74)

Scheme 4.30

Subsequent analysis of the proton NMR spectrum implied successful coupling of DTAF to the theophylline moiety (Figure 4.15). The spectra clearly shows the three parts of the structure-theophylline, linker and the fluorescein moiety.

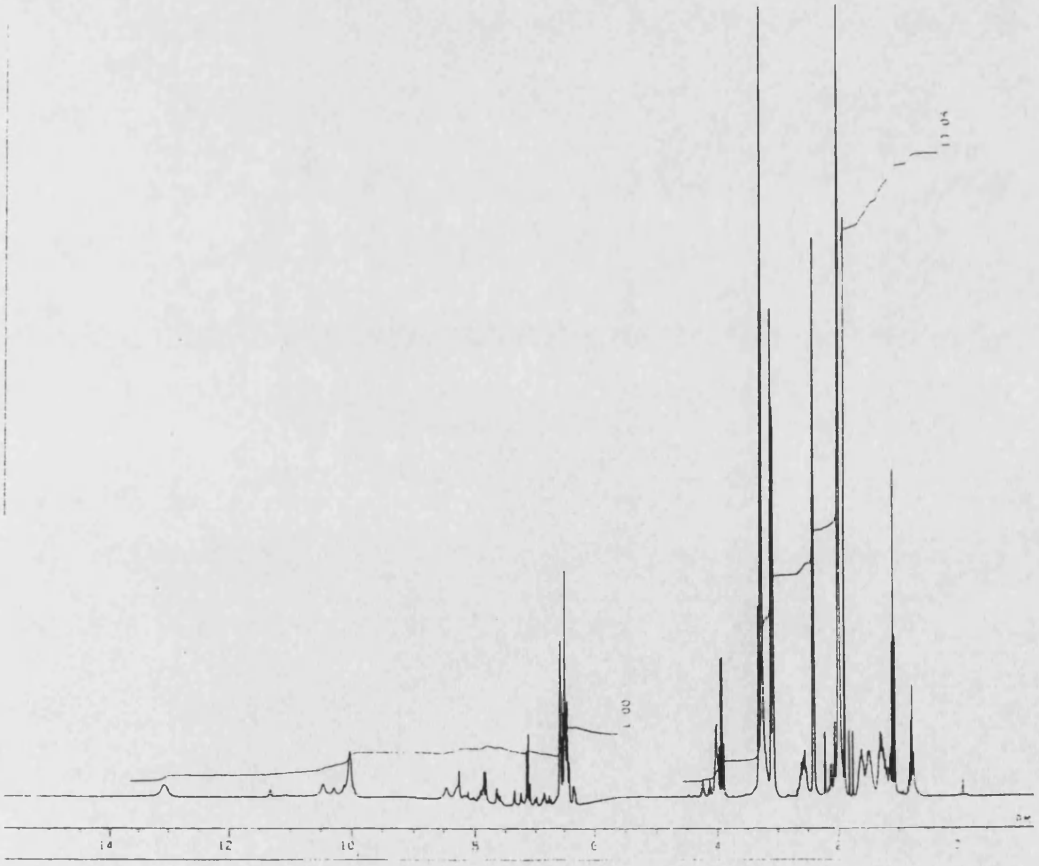


Figure 4.15.

As can be seen from Figure 4.15, the two N-CH_3 are found to resonate as singlets at δ 3.25 and 3.29 and appear slightly upfield *cf.* the N-CH_3 of the $(CH_2)_5$NHCSNH-linked fluorescein theophylline which appear at δ 3.24 and 3.4 respectively. The central $(CH_2)_5$ unit of the linker, again, appears as a broad peak centred at δ 1.29 while the other methylenes appear as discrete signals centred between the region δ 1.40-3.10.

In the fluorescein moiety the signal from the benzene ring and from the xanthene appear in separate clusters although only the Ar-3H of the benzene ring could be allocated with ease resonating as a doublet at δ 7.14. Further, the signals of the xanthene show symmetry of this group with the C-H signals indicative of a trisubstituted benzene. Thus, H-1' and H-8' appear as a doublet at δ 6.48 while the ortho coupled H-2' and H-7' appear as a dd at δ 6.41 with the C-H signal for H-4' and H-5' appear as a doublet at δ 6.56. The final assignment of the xanthene portion was attributed to resonance of two phenolic OH which appear as a singlet at 10.02.

It must be stated that the protons allocated for the xanthene portion are through comparison of both the fluoresceinthiourea derivatives synthesised (as for FITC derivative) and analysis of uncoupled DTAF in $(CD_3)_2SO$.

For further analysis a COSY spectrum is required although as is apparent in Figure 4.15, coupling of DTAF to the pentamethylene derivative has been both successful with isolation of final product (77) in good yield.

4.7 Conclusions

Initial approaches towards the synthesis of theophylline derivatives possessing a hydrophilic linker which could be coupled to a fluorescent congener were outlined in section 4.2. The first approach afforded 8-hydromethyltheophylline via two routes with the initial one-step procedure using the starting 5,6-diamino-1,3-dimethylpyrimidine precursor (14) proving to be of little use. The lack of success was attributed to a number of factors including the stability of the starting diamine and its lack of solubility in organic solutions.

Although successful utilisation of 5,6-diamino-1,3-dipropyl derivatives have been reported in the literature *via* use of the Traube route, their success has been attributed to their enhanced solubility due to the presence of the propyl group within the pyrimidine moiety.

Successful synthesis *via* use of the starting 6-chloro-1,3-dimethylpyrimidine was subsequently utilised in the final synthesis of the 8-alkylaminotheophyllines by reaction with α,ω-alkanediamines and, 8-alkylethoxyamine using 3,6-dioxaoctane-1,8-diamine (section 4.4.3).

Approaches to the synthesis of mono-protected polyether diamines proved to be disappointing although isolation of mono-protected Cbz-amino derivative of 3,6-dioxaoctane-1,8-diamine (35) was achieved in low yield. The lack of utilisation of polyether chains as hydrophilic linkers were attributed to the presence of the two ethoxy groups with possible deactivation effects causing lack of reaction.

Thus, from the initial synthetic strategies adopted a series of 8-alkylaminotheophyllines possessing an alkyl and an alkylethoxy linker, conjugated to FITC have been synthesised. Isolation of final target compounds (Figure 4.16) was achieved in good yields with coupling conditions stringently controlled at pH 9.0.

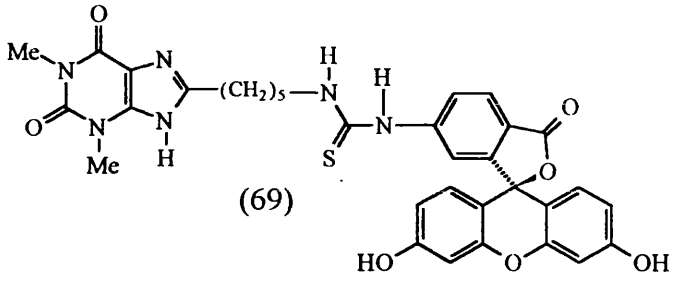
Through the successful coupling of FITC congener to the 8-alkylaminotheophylline precursor alternative fluorescent electrophiles were considered for conjugation. DTAF was a useful option in the respect that its quantum yield is comparable to fluorescein (approaching unity) with a larger Stokes shift as well as superior stability.

Coupling again was achieved under controlled basic conditions (pH 8.0) using the pentamethylene derivative as a model derivative, with isolation of target derivative (74) as an orange solid which was highly fluorescent in both organic and aqueous solutions.

Dansyl chloride was also coupled to the model pentamethylene derivative under basic controlled conditions (pH 9.5). The final product was isolated as a green solid which gave a green-blue fluorescence in both aqueous and organic solutions.

In summary the synthesis of 8-alkylamino derivatives of theophylline have been synthesised by careful choice of reaction conditions through adoption of the method of Fuschs *et al.*, 1977. The method can be applied to most α,ω-alkanediamines with isolation of final products in reasonable yields.

In utilising these 8-alkylaminotheophyllines, possessing an increasing methylene chain, successful coupling to fluorescent electrophiles has been achieved.



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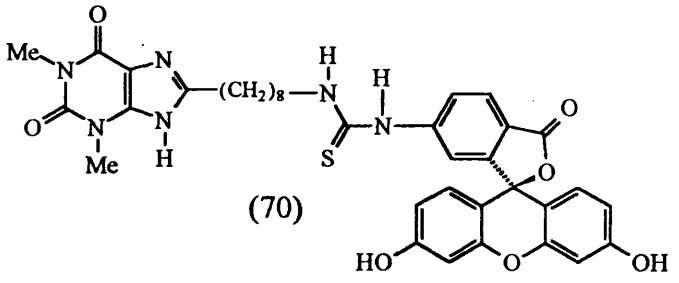

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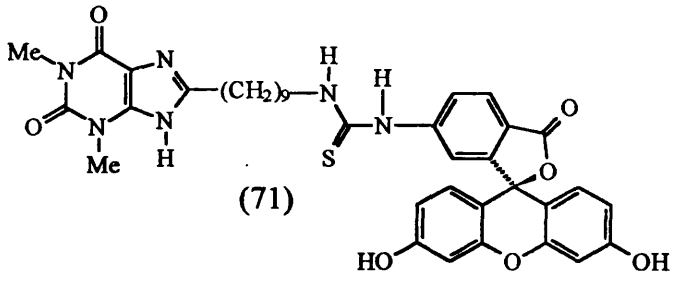

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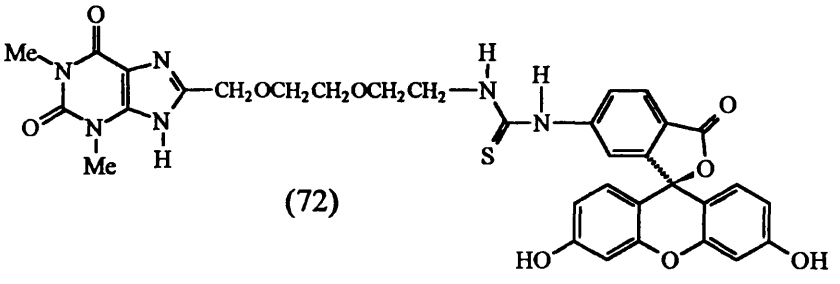


(72)

Figure 4.19

CHAPTER 5

BIOLOGICAL ASSAY

5.1. Introduction

The accurate and regular monitoring of serum theophylline levels is an important goal in clinical chemistry. The effective use of the drug is limited by its narrow therapeutic range and broad inter-patient variability attributed to its elimination kinetics which is influenced by such factors as age, smoking and liver function (see section 1.4.1).

Methods available for the quantitative measurement of serum theophylline levels include chromatographic techniques such as gas chromatography and HPLC and a variety of non-isotopic immunoassay techniques including EIA and FIA, these are detailed in section 1.5. Subsequently, the need for a rapid easy to use homogeneous immunoassay for theophylline has led to focus in the area of immunosensors.

Interest in immunosensors has increased rapidly in the past few years due to their many potential advantages including small size, speed of response and specificity (Christesen *et al.*, 1994). In particular immunosensors incorporating optical detection systems have been effectively utilised for monitoring a wide range of analytes including enzymes, hormones, viruses and blood components.

One such optical detection system which has shown great promise is the fluorescence capillary fill device (FCFD) an immunoassay system utilising a planar optical waveguide (Badley et al, 1987) for the fluorescent measurement of Rubella antibody (Parry *et al.*, 1990) and PSA (Fletcher *et al.*, 1993).

Sensitivities within the picomolar range have been recorded incorporating a variety of fluorophores (FITC, Rhodamine and Allophyocyanin).

The utilisation of a planar optical waveguide negates the need for a separation step *via* the use of an evanescent wave and is effective in the quantitative determination of antibody-antigen reactions (Badley *et al.*, 1987). The FCFD, thus offers the user high specificity and sensitivity with a rapid result time which ultimately can be used at the site of patient care.

In summarising the importance of monitoring serum theophylline levels and the need for a rapid easy-to-use homogeneous immunoassay, the FCFD was subsequently utilised for the development of an immunoassay for theophylline. In the previous section the assessment of the FCFD was discussed by synthesising fluorescent analogues of theophylline for use in a competitive immunoassay. These analogues were assessed for immunoreactivity by devising binding experiments using FCFD devices covalently immobilised with the anti-theophylline antibody. Subsequent reaction kinetics as well as a standard curve for theophylline were assessed with methodology and results outlined in the next two sections.

5.2. Materials and Methods

5.2.1 Reagents

All reagents were purchased from Aldrich-Sigma Chemicals (Poole, Dorset) unless otherwise stated.

Theophylline monoclonal antibody (rat anti-mouse IgA) was kindly donated by Serono Diagnostics, USA division.

All FCFD plates were manufactured at Serono Diagnostics, Woking.

5.2.2 Buffer solutions

Buffer solutions were as follows:

(i) Antibody buffer 0.1 M HEPES / 0.2 M NaCl;

(ii) wash buffer PBSTA pH 7.31;

(iii) Assay buffer Tris HCl pH 8.8 and BSA (1 mg ml^{-1}) in Tris buffer pH 8.8.

5.3 The Fluorescence Capillary Fill Device (FCFD)

The FCFD consists of two sheets of glass separated by a 100 μm gap with the lower surface containing the covalently immobilised antibody. The FCFD uses small sample volumes (25 μl) which as the device fills by capillary action negates the need for sample metering.

On completion of the immune reaction, a complex of the antibody and the fluorescently labelled analyte is formed on the lower surface of the device. Bound and free fluorescence are then discriminated by use of the evanescent properties of the device, thus eliminating the need for a wash or separation step. The basic structure of the FCFD is shown in Figure 5.

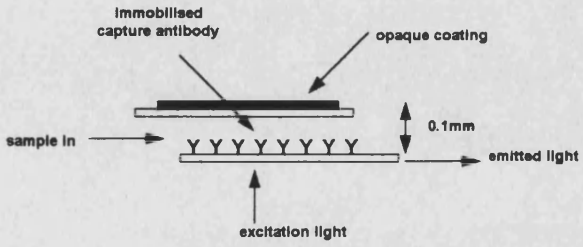


Figure 5. The FCFD (fluorescence capillary fill device)

5.3.1. Fabrication of FCFD devices

The FCFD devices were fabricated according to the method of Badley *et al.*, 1987 (Figure 5.1).

Pre-fabrication of devices involved prior washing of glass plates with water then acetone, followed by activation of lower base plate with γ-aminopropyltrimethoxysilane. Activation allowed covalent immobilisation of the anti-theophylline antibody to the lower plate with subsequent assembly of the device involving annealing of the top and lower plate under pressure.

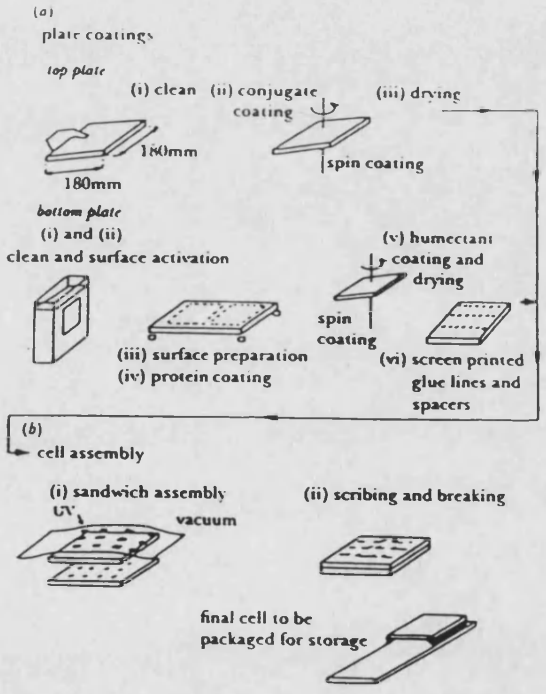


Figure 5.1. Schematic diagram of the FCFD fabrication process

5.3.2 Immobilisation of Antibody

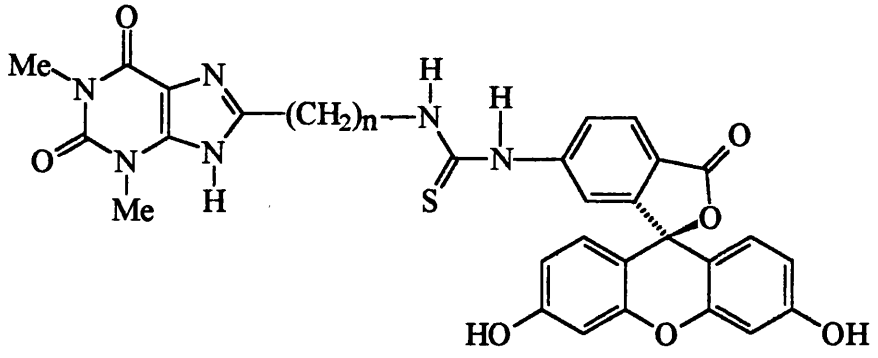
Anti-theophylline IgA mouse monoclonal antibody (4.41 mg ml^{-1}) was serially diluted to concentrations of 200 µg ml^{-1}, 100 µg ml^{-1}, 50 µg ml^{-1} and 25 µg ml^{-1} in 0.1 M HEPES / 0.2 M NaCl buffer. Coupling of the anti-theophylline antibody (15 µl) to the lower plate surface (activated) was through use carbonyldimidazole coupling chemistry.

Subsequent fabrication of the plates is outlined in section 5.3.1.

5.4 Conjugation of FITC to 8-alkylaminotheophyllines

8-alkylaminotheophyllines were synthesised in an analytically pure form as their hydrobromide salts as outlined in section 4.6.

Conjugation to FITC was performed under basic controlled conditions at pH 9.0 ± 0.1 using FITC isomer I. Isolation of the fluoresceinthiourea analogues involved purification through column chromatography to afford the desired derivatives (n=5, 8, 9 and alkylethoxy) as orange solids (Figure 5.2).



n = 5, 8, 9, $CH_2OCH_2CH_2OCH_2CH_2$

Figure 5.2. General structure of the fluoresceinthiourea derivatives synthesised

All fluoresceinthiourea derivatives were subsequently dissolved as stock solutions either in Tris HCl buffer pH 8.8 or Tris buffer containing BSA (1 mg ml^{-1}). Sequential dilutions were performed by addition of either Tris buffer or BSA solution.

5.5 Assessment of assay performance

All assays using the FCFD were performed in duplicate following incubation at room temperature for 20 min.

Measurement of signal within the FCFD was through use of a fluorimeter equipped with appropriate filters for the FITC chromophore, with subsequent signal being inversely proportional to analyte concentration.

5.5.1 Evaluation of immunoreactivity

The immunoreactivity was assessed through construction of a dose-response curve using the nonamethylene fluoresceinthiourea derivative which was serially diluted at appropriate concentrations in the assay buffer.

Following incubation, a plot of signal against analogue concentration (Figure 5.3) was constructed by measuring the signal from devices containing an increasing concentration (1×10^{-12}-0.5×10^{-6} M)of the labelled analogue.

5.5.2 Assessment of antibody loading

Devices were fabricated using four antibody loading concentrations between 200 μg ml^{-1} and 25 μg ml^{-1}.

The effect of antibody loading in relation to the total binding of the fluorescently labelled analogue was investigated by incubating devices, at appropriate dilutions of analogue with increasing concentration of antibody immobilised on surface. Following incubation, devices were measured and the signal recorded.

The influence of antibody concentration on the signal observed as a measure of total binding to solid phase is depicted in Figure 5.4 as a plot of signal against analogue concentration.

5.5.3 Reaction kinetics

Incubation time was determined by studying the kinetics of theophylline-FITC binding to anti-theophylline solid phase. Three concentration pools of unlabelled theophylline (50 μg ml^{-1}, 25 μg ml^{-1} and 7.5 μg ml^{-1}) were used to assess the competitive binding reaction occurring within the devices. An appropriate concentration of the pentamethylene theophylline analogue (1 μM) was chosen, from the specific binding isotherm which was due solely to specific signal, for assessment of binding kinetics. Incubation times of 5, 10, 15, 20, 25 and 30 min were evaluated at 30 sec intervals. From the graph of signal against time (Figure 5.5), the optimal incubation time was chosen (*e.g.* time to reach equilibrium). Incubation times were thus set at 20 min.

To assess the kinetics of binding due solely to the FITC analogue, devices were incubated with a known concentration of the analogue and signal measured at 30 sec intervals for a time of 30 min. Figure 5.6 depicts the kinetics of binding of the FITC analogue without interference from theophylline.

5.5.4 Measurement of total binding

FCFD devices coated with 200 μg ml^{-1} of antibody were used in the construction of binding isotherms for each of the fluoresceinthiourea analogues synthesised. In assessing total binding increasing concentration of analogue (0-5 μM) was introduced into devices. After incubation, total binding isotherms for each analogue was constructed by plotting signal observed against analogue concentration (μM).

A plot of signal against concentration for each of the analogues (n=5, 8, 9, alkylethoxy) gave a typical dose response curve, see Figures 5.7 - 5.10.

5.5.5 Measurement of Specific Binding

Following construction of total binding isotherms, the specific binding of each analogue to solid phase was assessed using a competition binding assay between the labelled theophylline derivatives and unlabelled theophylline present as inhibitor.

Devices were incubated with various concentrations of the analogues, serially diluted from stock solution (0-5 μM), together with a 1000 fold molar excess of theophylline concentration.

Following incubation, the degree of non-specific binding (NSB) was assessed with specific binding calculated by subtraction of NSB from Total Binding

Specific binding = Total binding - Non specific binding

Specific binding of each analogue to immobilised solid phase is outlined in Figures 5.7-5.10, with linear regression analysis providing a measure of the binding affinities (K_a) of each fluorescent analogue for the anti-theophylline antibody (Table 5).

The analogue having the highest affinity for the anti-theophylline antibody was subsequently chosen as the labelled analyte for use in the theophylline competitive assay.

5.5.6 Theophylline standard curve

After preliminary optimisation of the assay conditions:

(a) for the antibody loading concentration giving reasonable signal

(b) for antigen concentration giving reasonable response to antibody

(c) for the kinetics of the assay and thus the time for assay to reach equilibrium

(d) for assessment of specific binding, with the analogue chosen as having the highest affinity for the anti-theophylline antibody

a standard curve for theophylline was constructed.

The assay involved use of devices coated with antibody concentration of 200 μg ml^{-1} with the chosen analogue, the pentamethylene derivative at a concentration of 1 μM. This concentration was chosen from analysis of the specific binding isotherms and observing a concentration where signal is due only to analogue and background effects are minimised, Figures 5.12.

Theophylline standards were dilutions of stock between the clinically relevant range of theophylline (0-50 μg/ml) (0-277 μM) as well as the non-clinical and the highly toxic range (1 nM-10 mM). Thus standards curves using the pentamethylene analogue as the labelled analyte were assessed in both buffer (Tris HCl, pH 8.8) and BSA solution.

Standard curve for theophylline in Tris buffer is depicted in Figure 5.12 together with the inhibition profile at high and low levels of theophylline concentration, Figure 5.13.

5.5.7 Specificity

The cross-reactivities of various analogues of theophylline including the structurally related methylxanthines (caffeine and theobromine) as well as several metabolites of theophylline were assessed at physiological concentrations (50 μg ml^{-1}) and are summarised graphically in Figure 5.12.

5.5.8 Assessment of precision

Three pools of the selected concentration of the labelled analyte (pentamethylene derivative) were assayed as 10 replicates in one assay (within-run precision) and for a period of 10 different days (between run).

Thus within-assay coefficients of variation (CVs) were 6.5, 6.0 and 5.2 % at mean theophylline concentration of 5, 10, and 25 μg ml^{-1} respectively. Between assay are slighter higher at 8.0, 7.5 and 5.0 % at these levels.

5.6 Results

Figure 5.3 shows a typical dose-response curve of log concentration against signal and as can be seen the curve is not quite sigmoidal indicating that signal could be measured at higher concentrations.

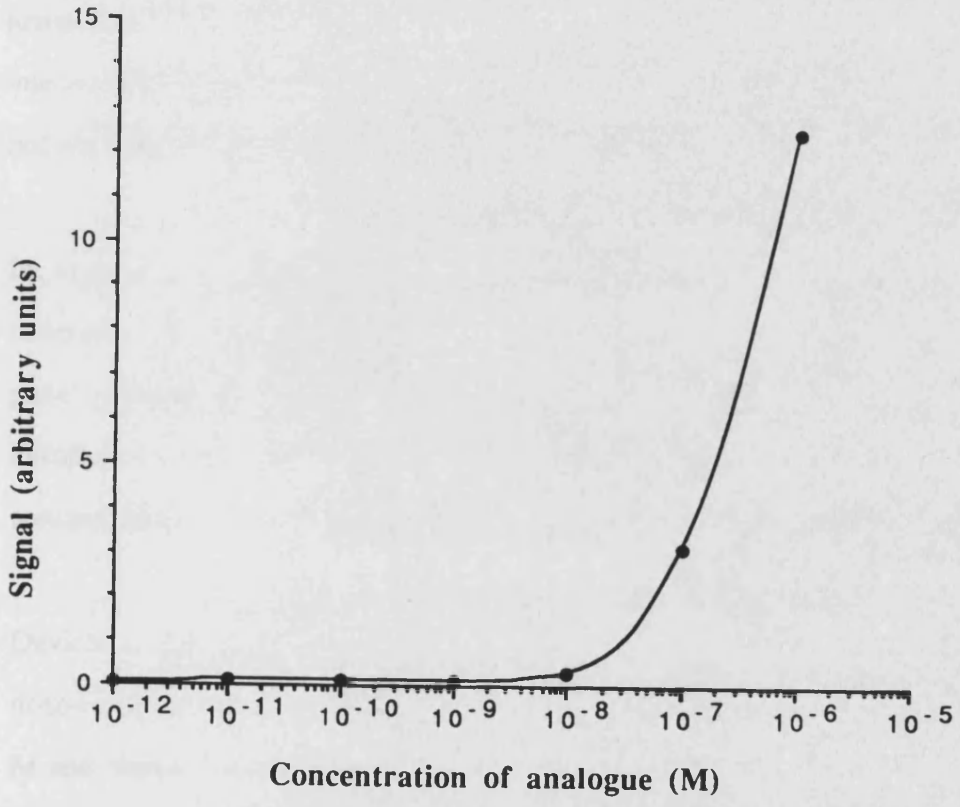


Figure 5.3. A dose-response curve for the pentamethylene analogue in the presence of Tris HCl buffer pH8.8.

However, increasing the analogue concentration above 1 μM required lowering of the gain of the FCFD analyser with an increase in scatter at high concentrations.

As can be observed from Figure 5.3, at low analogue concentrations from 10^{-12} to 10^{-9} M, there is little change from base line values. Increasing the analogue concentration from 10^{-9} to 10^{-7} M appears to follow a steady increase in signal which rises rapidly from 10^{-7} to 10^{-6} M and beyond. A plateau of the signal was not observed although lowering of the gain could be one solution. However, the problem of non-specific interactions appears to be more prevalent at high analogue concentrations, which are not apparent at low concentrations where background effects appear to be lower.

In assessing the effect of antibody loading, devices immobilised with increasing concentrations of antibody solution were used as titres for optimisation of the base plate covering. A range of concentration of the FITC-analogue (0-2 x 10^{-6}M) was introduced into the devices containing a sequential decrease in antibody concentration.

Devices coated with 200 μg ml^{-1} and 100 μg ml^{-1} of antibody solution gave good dose-response curves with saturation of signal being attained at concentrations of 1 μ M and above, Figure 5.4. However it was surprising to observe that devices coated with 50 μg ml^{-1} antibody solution also gave good signal to concentration ratio. In fact, from Figure 5.4, the dose-response curve for this concentration is comparable to the devices containing higher coating concentrations of antibody solution.

However, devices containing the lowest concentration of antibody solution gave comparably lower signals with increase in analogue concentration, implying the limiting effects of the antibody solution.

Subsequently, the performance of the FCFD assay including assessment of the reaction kinetics, the construction of binding isotherms, as well the standard curve and precision of the assay were performed in devices immobilised with 200 μg $ml^{-1}$of anti-theophylline antibody. This in effect ensured that the antibody coating concentration was not the limiting factor in the assay, particularly in establishing a standard curve for theophylline using competitive binding.

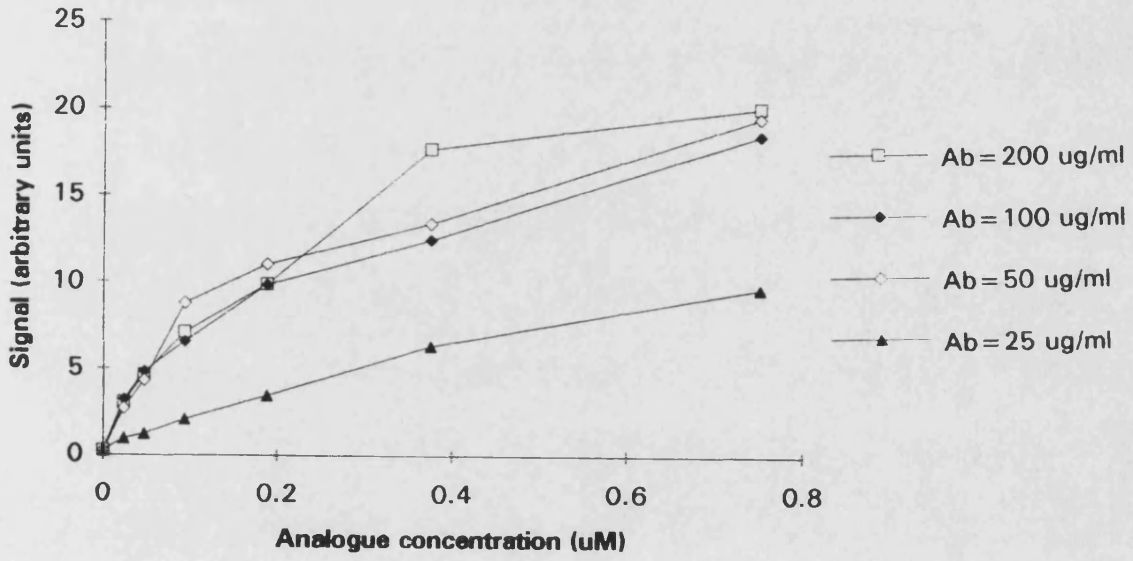


Figure 5.4. Evaulation of antibody loading

The kinetic performance of the assay using a standard concentration of the labelled analogue (pentamethylene derivative) in the presence of unlabelled theophylline is depicted graphically in Figures 5.5. As is apparent from Figure 5.5, the initial rate of reaction in the presence of low concentrations of theophylline (7.5 μg ml^{-1}) is extremely rapid with equilibration of the reaction around 5 minutes with the signal appearing to plateau after 15-20 min into the assay. At high analyte concentrations (25 μg ml^{-1} and 50 μg ml^{-1}) the signal observed is not only lower but equilibration of the reaction appears to occur at 10 min into the reaction, after which time a plateau of the signal is observed.

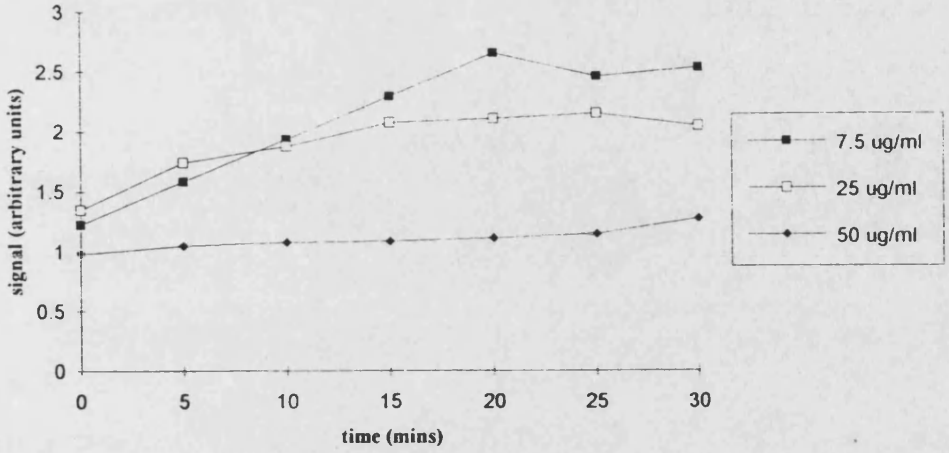


Figure 5.5. A comparison of the kinetics of binding in the presence of three standard concentrations of theophylline and the pentamethylene analogue at 1μM concentration

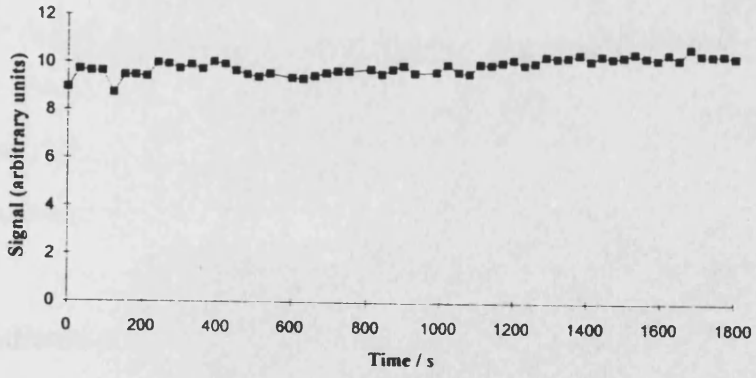


Figure 5.6. Kinetics of binding due to the pentamethylene analogue at 1 μM concentration.

In comparison devices containing labelled theophylline without the presence of inhibitor seem to equilibrate extremely rapidly, within the first 2-3 min of the reaction, Figure 5.6. After this initial increase the signal seems to plateau and remains steady up to 20 min into the assay.

From these observations it is apparent that the reaction kinetics of the competition assay is complicated, subsequently, with low concentrations of analyte the assay is rapid with little change in the signal observed. As this concentration is increased from 25 $\mu g\ ml^{-1}$ to 50 $\mu g\ ml^{-1}$ the rate at which equilibrium seems to occur increases with a lowering of the signal observed. This increase in the time for equilibration to occur is attributed to the competition process occurring between labelled theophylline and unlabelled theophylline for a limited number of antibody binding sites within the devices. Overall an incubation time of 20 min seems to ensure that the competitive binding reaction between labelled theophylline and theophylline in sample has reached equilibrium and standard curve can be constructed with ease.

Binding isotherms were constructed by assessing both the total and the non-specific binding of each analogue to the anti-theophylline antibody in Tris buffer. Subtraction of total from the non-specific signal gave the corresponding specific binding isotherms of each of the analogues assessed. The concentration was optimised between 0 and 5 μM were maximum signal was obtained for an increase in analogue concentration.

From the isotherms constructed each analogue gave a typical saturation plot of signal against concentration, Figures 5.7-5.10. Overall saturation of signal appears to occur between 1-2 μM for each analogue, with the pentamethylene spacer giving the highest signal to concentration ratio.

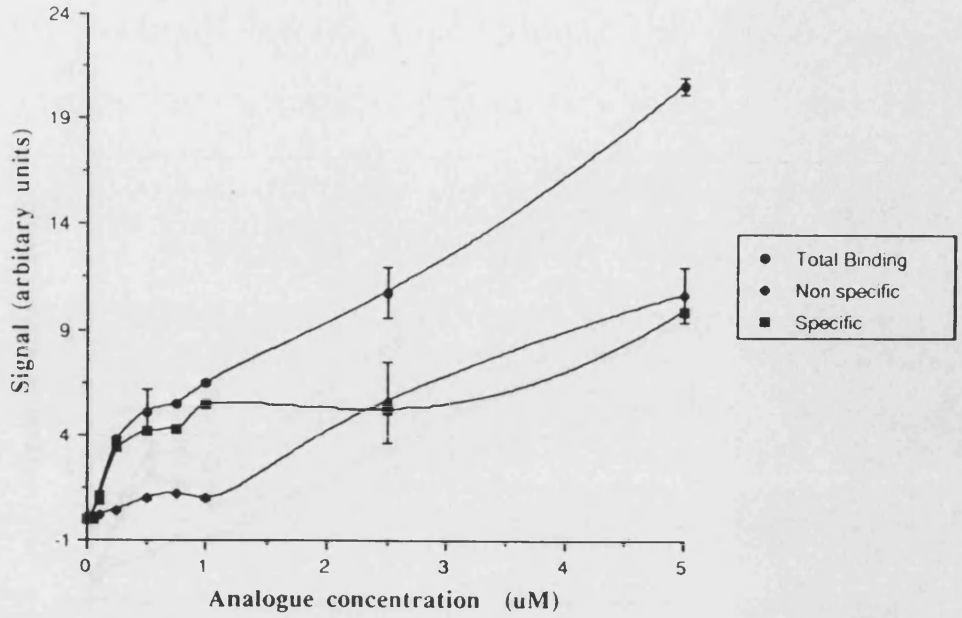


Figure 5.7. The binding isotherms of the pentamethylene analogue (n=5) in Tris HCl buffer at pH 8.8

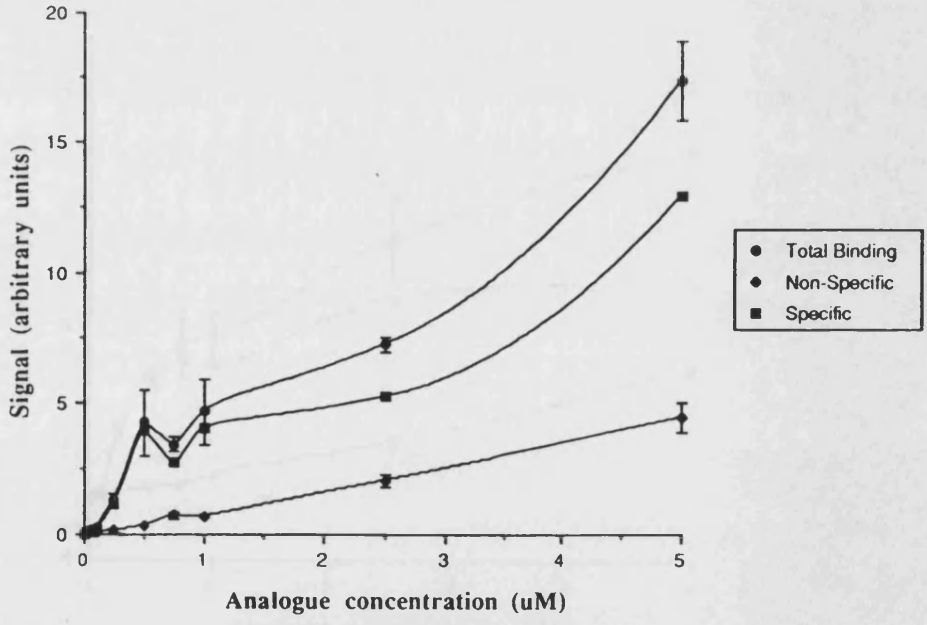


Figure 5.8. The binding isotherms of the octamethylene analogue (n=8) in Tris HCl buffer at pH 8.8

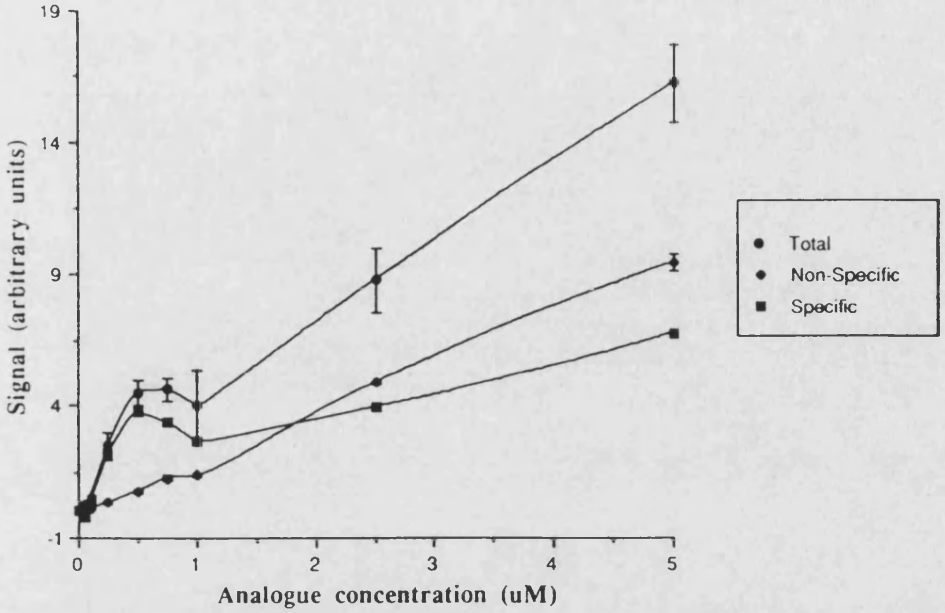


Figure 5.9. The binding isotherms of the nonamethylene analogue (n=9) in Tris HCl buffer at pH 8.8

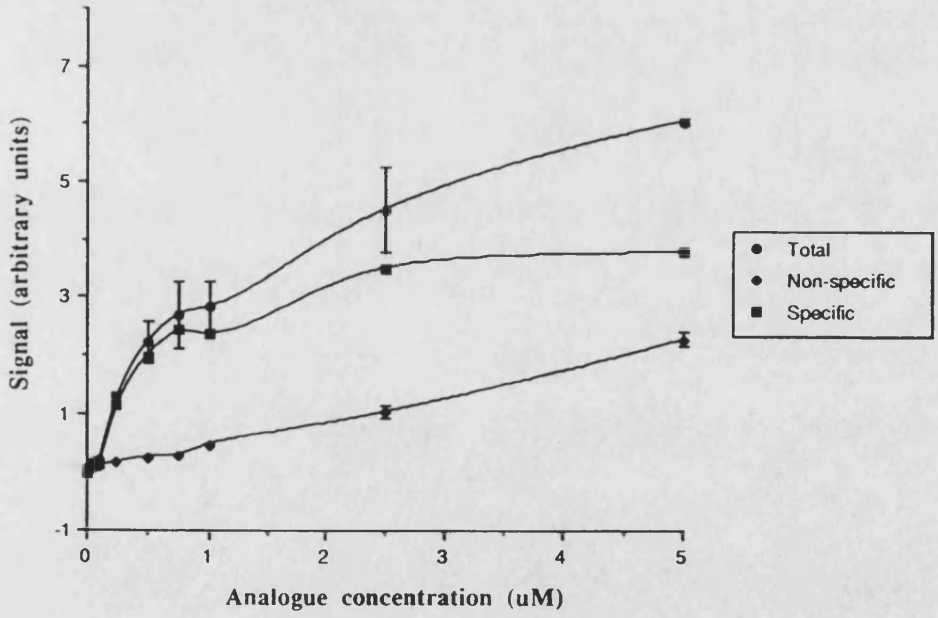


Figure 5.10. The binding isotherms of the alkylethoxy analogue in Tris HCl buffer at pH 8.8

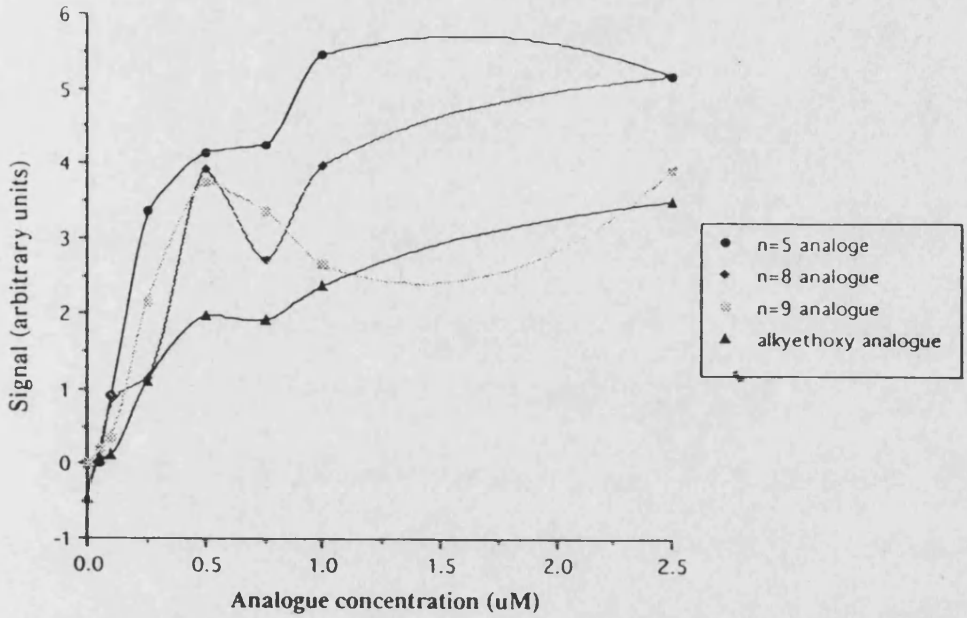


Figure 5.11. A comparison of the specific binding isotherms of the fluorescein thiourea derivatives synthesised

In each case the total binding gave the highest signal to concentration ratio while the non-specific binding curves gave a much lower almost linear plot. Subsequently, for each analogue concentrations below 1 μM gave specific signal without interference from non-specific background effects.

A comparison of the specific binding of each analogue against concentration is depicted in Figure 5.11. As can be observed the pentamethylene spacer gave the highest signal with increase in analogue concentration, with both the octa and nonamethylene derivatives giving comparable results below 0.5 μM. While the lowest response was attributed to the alkylethoxy spacer.

These results agree fairly well to the rationale of the similarity in structure of each analogue synthesised. As would be predicted, binding isotherms would be fairly similar with the affinity of each analogue calculated through a Lineweaver-Burke plot, Table 5.

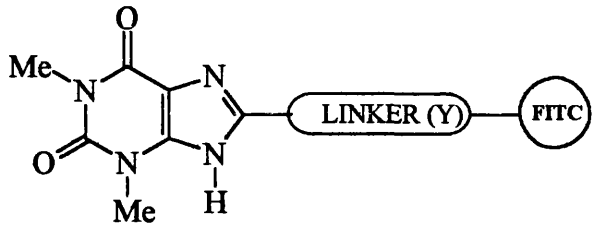


Table 5. The Binding Affinities of the Fluorescein-thiourea derivatives

Linker moiety (Y)	Binding Affinities Ka L mol^{-1}
$(CH_2)_5$	$3.09 \pm 1.5 \times 10^6$
$(CH_2)_8$	$1.40 \pm 0.66 \times 10^6$
$(CH_2)_9$	$3.23 \pm 1.82 \times 10^6$
$CH_2OCH_2CH_2OCH_2CH_2$	$1.04 \pm 0.37 \times 10^6$

Analysis of binding affinity data infers a low affinity (μM) of each analogue for the anti-theophylline antibody. The highest affinity Ka = 3.08×10^6 L mol^{-1} was attributed to the pentamethylene analogue, although each analogue gave comparable affinity constants, see Table 5.

From the calculation of the binding affinity, the pentamethylene spacer was selected as the derivative for use in assessing the full performance of the FCFD theophylline assay.

Subsequently, a standard curve for theophylline was constructed with the basis of the assay the competitive binding reaction between the labelled theophylline derivative and unlabelled theophylline standards for the limited number of antibody binding sites within the lower base plates of the devices.

Assays were performed in both Tris buffer (pH 8.8) and BSA solution, which provided a comparable protein matrix. The standard curve for theophylline is depicted in Figure 5.12, as a plot of concentration against signal. As can be observed an increase in theophylline concentration results in a decrease in signal, which is attributed to the competition process occurring within the devices. Increasing the concentration of theophylline standard to 100 μM and above results in a typical inhibition profile. Further, expansion of the concentration range of the theophylline standards resulted a typical inhibition profile as depicted in Figure 5.13.

From Figure 5.13, it is apparent that at low concentrations of theophylline (10^{-8}M) high signals are observed which appear to scatter until 1 μM (10^{-6}M) where upon there is a linear decrease in signal with increasing theophylline concentration. This linear decrease results in a lowering of the signal measured up until 30 μM, where signal appears to plateau. This inhibition profile subsequently, covers the clinical range of theophylline and allows discrimination between low and toxic levels of theophylline in sample.

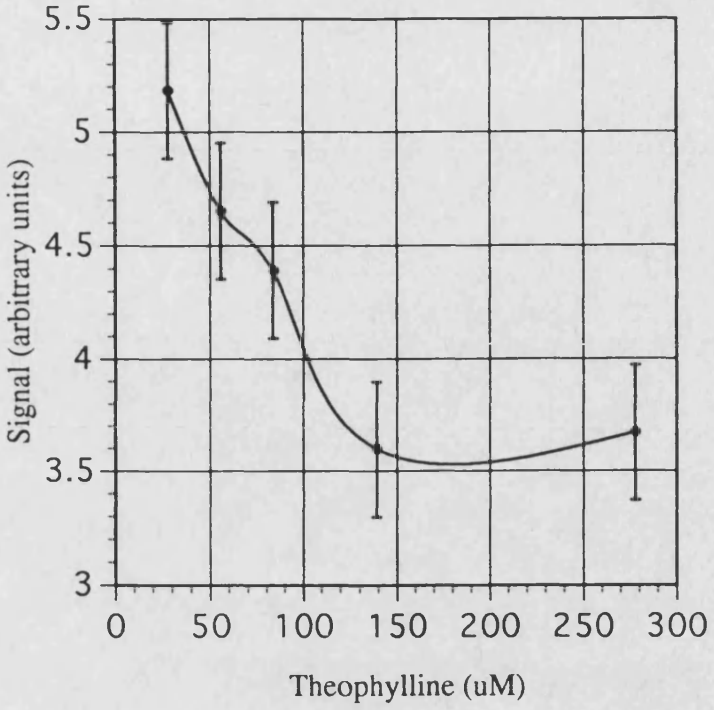


Figure 5.12. A standard curve for theophylline

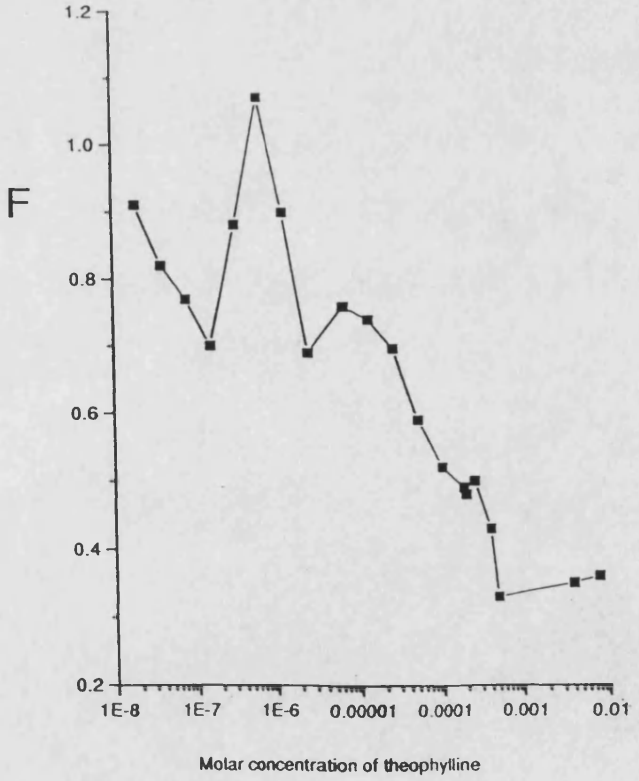


Figure 5.13. A competitive inhibition profile for theophylline in the presence of labelled analyte

Further, as a comparison theophylline inhibition profiles have also been performed in BSA solution covering this large concentration range, Figure 5.14. The graph depicts two profiles for the inhibition curve, one at 20 min incubation time and the other at 30 min incubation. The profile is closely related to Figure 5.13, although in the presence of BSA there appears to be less scatter at high concentrations and can be attributed to bulk interaction in sample. The assay clearly shows that at 20 min an inhibition profile of theophylline concentration in BSA solution can be constructed depicting the safe and toxic levels of theophylline in sample. Increasing incubation times gives a slightly better profile and a smoother curve.

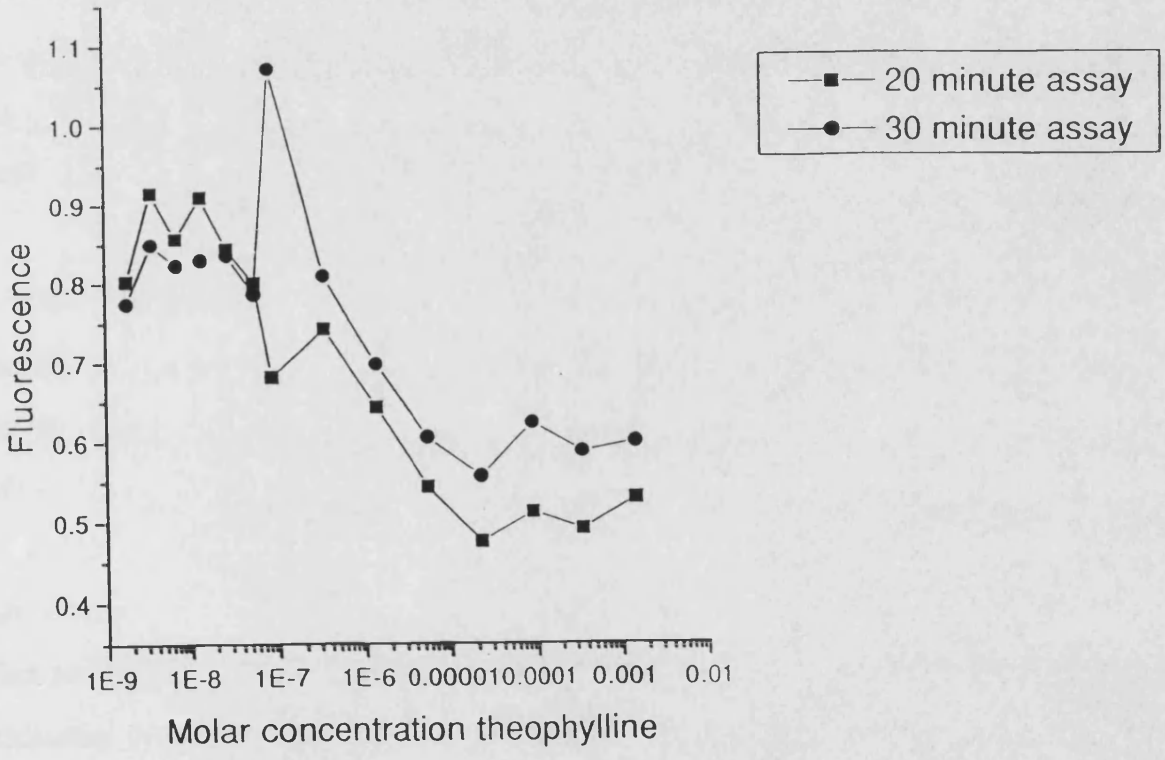


Figure 5.14. A competitive inhibition profile of theophylline in the presence of labelled analyte in BSA solution

The specificity of the assay was determined by evaluating the cross reactivity of the theophylline antibody in the presence of structurally related xanthines and metabolites of theophylline, Figure 5.15. The cross reactivity of these analogues was assessed by performing inhibition studies in the presence of anti-theophylline antibody at physiological concentrations of inhibitor (50 $\mu g\ ml^{-1}$).

As can be observed from Figure 5.15, the structurally related xanthines caffeine and theobromine, as well as 1,3-dimethyluric acid, gave no appreciable difference in signal over the required concentration range and therefore did not cross react with the anti-theophylline antibody. Further, this effect is particularly apparent for 1-methylxanthine and 3-methylxanthine where very little signal change is observed within this concentration range in comparison to theophylline. Thus, no significant cross reactivity was observed for these metabolites at physiological concentrations with the assay being highly specific to theophylline.

As a final assessment of assay reproducibility precision studies were performed using ten replicates of labelled theophylline analogue in the presence of three standard concentrations of theophylline. The CVs for the within run precision were fairly low with an average of 5%, with low concentration of the analyte giving a slightly higher CV and implying more scatter with errors in signal measurement. In comparison at high concentration of analyte (25 $\mu g\ ml^{-1}$) the CV appears lower which may be due in effect to the competition process occurring within the devices and subsequently a stabilisation of the signal.

The day to day precision appeared to be higher with results on alternative days giving more scatter in the data observed than in the day to day running of the assay. Again, devices with high analyte concentration gave a lower CV in comparison to lower concentration and again this was attributed to competition within the assay.

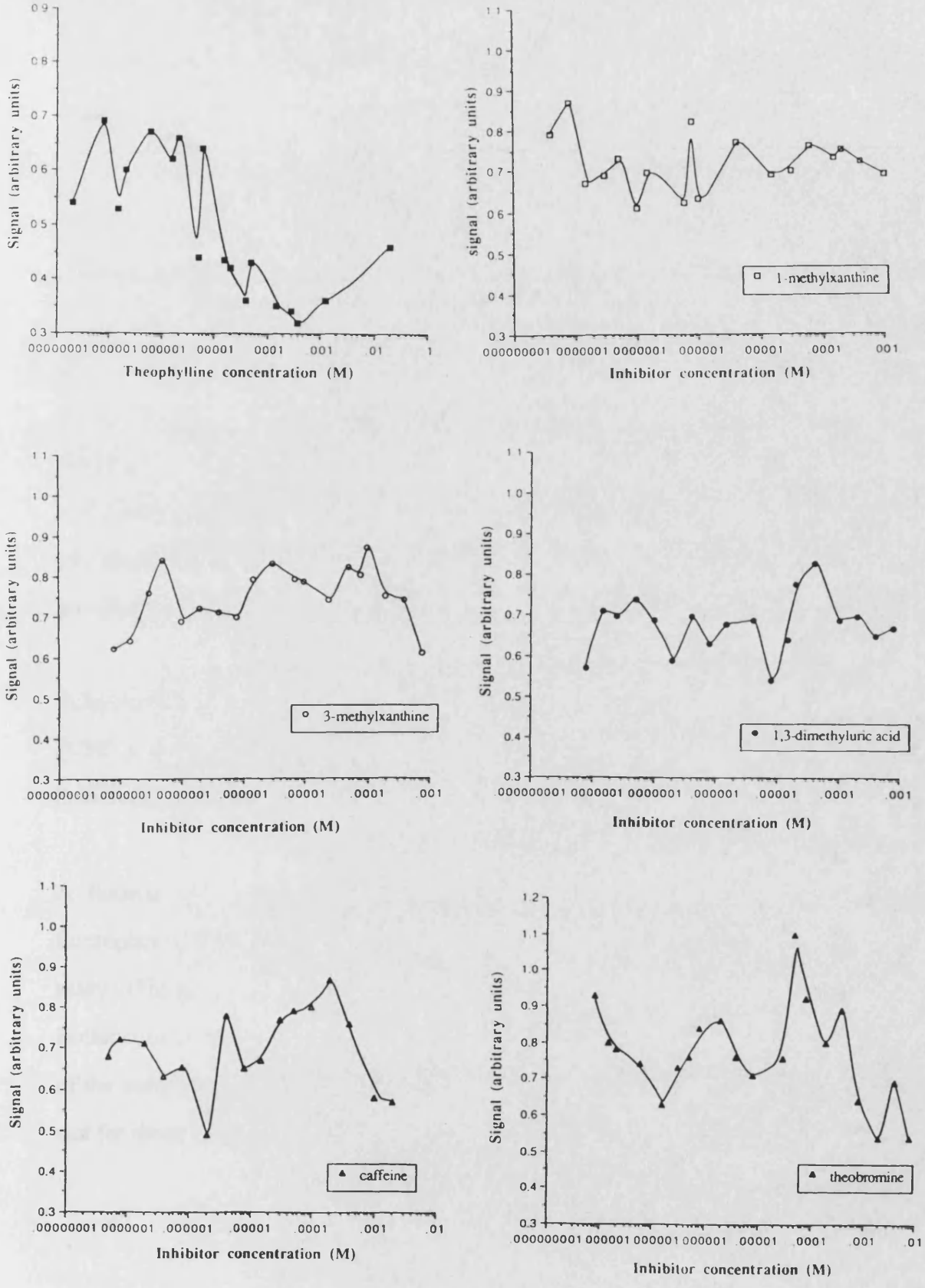


Figure 5.15. Inhibition studies of structurally related xanthines for assessment of cross-reactivity.

5.7 Discussion

The development of a novel immunoassay for theophylline using the FCFD requires careful optimisation of the assay conditions. Initial requirements of the assay were:

(i) a rapid assay time (less than 20 min);
(ii) specificity, with no interaction with exogenous and endogenous derivatives of theophylline including the structurally related xanthines caffeine and theobromine, as well as theophylline metabolites such as 3-methyluric acid and 3-methylxanthine;
(iii) good precision giving consistent results during multiple assay runs as well as providing a standard curve within the clinical range of theophylline.

Subsequently, in developing a competitive immunoassay for theophylline utilising the FCFD a series of theophylline analogues incorporating an alkyl and an alkylethoxy linker conjugated to FITC were synthesised as outlined in section 4.6.

A fluorescence based assay was developed due to the successful utilisation of fluorophores in the FCFD assay including the use of APC (allophyocyanin) in a PSA assay (Fletcher *et al.*, 1993, O'Neill *et al.*, 1995) and FITC (fluorescein isothiocyanate) in a rubella assay (Parry *et al.*, 1990). Subsequently, for measurement of the small haptens, in particular theophylline, the FCFD was chosen as a qualitative test for theophylline.

However, theophylline itself is problematic in that (i) it is a very small hapten having a Mr of 180.1 and thus needs to be conjugated to large protein complex in order to obtain a specific monoclonal (ii) is very hydrophobic owing to the presence of the two N-Me groups which can hydrogen bond (section 1.1) to neighbouring theophyllines.

Reaction conditions of most immunoassays are favoured at neutral to reasonably high pH (i.e. pH 7.4-9.0) implying that theophylline as well as the labelled theophylline analogues needed to be soluble in the assay buffer favoured. Ultimately the aim of the assay was to achieve a standard curve in plasma serum and whole blood using patient samples, however serum albumin was the only protein matrix assessed in the standard curve. Thus correlation with an existing method couldn't be performed until an accurate and sensitive assay could be optimised.

Subsequently, in evaluating an assay for theophylline utilising the FCFD, binding isotherms were set up for each fluorescent analogue synthesised, structure of analogues are depicted in Figure 5.2.

This was not only to assess the immunoreactivity of each analogue but also to ascertain the binding affinities of each analogue for the anti-theophylline monoclonal antibody immobilised within the devices.

As outlined in the section 5.5, all analogues were immunoreactive against the monoclonal antibody and gave typical binding isotherms (*e.g.* increase in signal for increase in analogue concentration).

Saturation of the immobilised antibody appears to occur at a concentration of 1 μM both in terms of total and specific signal observed.

Above this concentration only the pentamethylene and the alkylethoxy analogue (Figures 5.7 and 5.10 respectively) appear to show signs of plateauing particularly for the specific signal observed. For the octamethylene and nonamethylene analogues (Figures 5.8 and 5.9 respectively) the total and specific signals observed appear to rise rapidly above 2.5 μM with no plateau being observed for analogue concentration up to 5 μM.

This increase (enhancement) in the signal fluorescence observed is a real effect and can only be attributed to the increase in analogue concentration which causes some physical effect in the FCFD. From the stochiometric equation it is apparent that saturation of the antibody binding sites, immobilised within the FCFD, occurs at an analogue concentration of 1 μM. Above this concentration there appears to be an excess of analogue within the device and, so as observed background effects are high. However, this rapid increase in signal fluorescence, particularly for the long chain alkylamino derivatives (n = 8, 9) appears to indicate some form of interaction of neighbouring xanthene fluorescent moieties away from the immobilised antibody site leading to the possibility of stacking of these planar molecules and, hence, an increase in signal fluorescence observed. This observation is particularly true for the total signal measured where there is the greatest enhancement of the signal observed.

The specific signal observed (see Figures 5.7-5.10) apears to show a smaller enhancement in fluorescence signal which is attributed to the fact that specific signal is calculated from both the total and non specific signals measured within the FCFD devices.

Subsequently, the non specific signal was also assessed and found to be detrimental above concentrations of 2 μM (see Figures 5.7-5.10) where there is a distinct lack of selectively between the specific signal obtained and background effects.

Therefore, in calculating the binding affinities of the fluorescent analogues, it was found that all the analogues had similar affinities for the theophylline antibody within the μM range.

Inhibition profiles utilising the pentamethylene analogue gave reasonable competitive binding curves through a broad concentration range in the presence of Tris buffer (pH 8.8), Figure 5.13 and BSA solution, Figure 5.14.

Thus, the FCFD provides an assay for theophylline in the range of clinical interest (1-50 μg ml^{-1} = 5.56 - 278 x 10^{-6}M) without interference from other exogenous or endogenous xanthines at their normal physiological concentration, Figure 5.15. However, the only problem encountered in the assay seemed to be the lack of discrimination of signal from label bound to solid phase in relation to background at high concentrations. This may in fact be attributed to too little antibody present within the devices, even at high concentrations of antibody (200 μg ml^{-1}) present. Although only 15 μl is immobilised onto solid phase, a larger volume may be required for further utilisation of the FCFD.

Further, in assessing the kinetics and the precision of the assay, it was found that the competitive reaction required incubation times of less than 20 min which ensured equilibration of the reaction had occurred. Also, the reasonable precision of the assay, particularly between every run ensured that the assay was robust and that results were reproducible.

However, further assessment of the assay needs to be fully investigated in order to develop a suitable assay for theophylline, in particular:

(a) assaying of theophylline in a suitable protein matrix such as plasma serum with a view to develooping in assay in whole blood. Problems may be encountered due to the "stickiness" of the blood as well as the problems associated with the binding of plasma serum to theophylline which appears to be between 40-60% (Brors *et al.*, 1983, Buss *et al.*, 1983);

(b) the sensitivity of the assay has to be determined together with the lowest limit of detection;

(c) a change in the immobilised antibody which is an IgA fraction and exists as a large complex. The use of an IgG fraction raised against theophylline or one of the analogues synthesised would ensure a higher affinity of the label for the antibody. Ultimately an affinity constant in the nanomolar range would be useful ensuring reasonable competition between theophylline and the labels. These options have to be fully investigated.

(e) A final but the most crucial aspect of the assay, a correlation with an existing method would be a useful comparison. Existing assays would provide an insight into the effectiveness of the FCFD for measuring clinical samples spiked with theophylline.

5.8 Conclusion

Overall, an assay for theophylline covering the clinical range has been achieved with a reasonable reaction time which is both highly specific and precise. The assay developed, although not commercialised, has the potenial to distinguish both safe and toxic levels of theophylline in assay buffer.

Ultimately for a quantitative assay the FCFD system has to be fully optimised (section 5.7) with a view to a commercial device incorporating all the components of the assay, the antibody as well as the fluorescent label in a rapid easy-to-use homogeneous assay.

Realistically, the device although simple to use requires a decrease in its reaction time as well as minaturisation of the technology in order to be of use in the clinical environment. In particular such technology can be envisaged for use in an emergencency situation where rapid results are required and on the spot treatment essential.

EXPERIMENTAL

I.R. spectra were recorded on the Perkin-Elmer 782 spectrophotometer as potassium bromide discs unless otherwise stated.

^{1}HNMR spectra were recorded using Jeol JNM GX270 and Jeol JNM EX400 spectrometers at 270 MHz and 400 MHz, respectively. Chemical shifts (δ) were measured in ppm relative to tetramethylsilane (TMS) which was used as internal standard. Values of the number of protons for each signal are given between the brackets. Multiplicities are indicated as follows s (singlet); d (doublet); dd (double doublet); t (triplet); dt (double triplet); q (quartet); m (multiplet), br (broad).

Mass spectra were obtained using VG7070E analytical mass spectrophotometer, University of Bath, and reported in the form m/z (intensity relative to base = 100) for selected ions. Spectra were obtained using electron impact (E.I.), chemical ionisation (C.I.) or fast atom bombardment (FAB) ionisation techniques.

Elemental analysis was carried out using the EMA system 1106, microanalysis unit, University of Bath.

Melting points (m.p.s) were measured using a Gallenkemp melting point apparatus and are uncorrected.

All reactions were monitored by T.L.C on pre-coated silica plates manufactured by Merck; (Merck t.l.c aluminium sheets) silica 60 F254 Art no 5635.

Products were visualised in a variety of ways; ultraviolet (U.V.) light; iodine vapour; spraying with phosphomolybidic acid in methanol followed by heating; ninhydrin in ethanol. TLC system of dichloromethane : methanol was used, unless otherwise stated, with all spots being detected by fluorescence quench at 254 nm.

Chromatographic separations were carried out using Sorbsil C60 (0.040 - 0.063) silica gel in the noted solvent system using forced air.

All solvents were evaporated under reduced pressure.

When required, ethanol was dried over magnesium turnings and iodine, collected after distillation.

Light petroleum refers to the fraction with boiling point 60-80°C.

Organic phases were dried over magnesium sulphate ($MgSO_4$) unless otherwise stated.

1,3-Dimethyl-3,7-dihydro-8-hydroxymethyl-1H-purine-2,6-dione (15)

Hydroxyacetic acid (8.5 g, 112 mmol) was added to 1,3-dimethyl-5,6-diamino-pyrimidine-2,4-dione (8.3 g, 49 mmol) and the two compounds were thoroughly mixed until a brown paste was seen. The mixture was then heated at 100°C for 1 h, after which time the solution was diluted with aqueous NaOH (1 M, 110 ml) and was boiled under reflux for a further 2.5h.

The resulting solution was then acidified to pH 4 with 10% aqueous H_2SO_4, cooled to ambient temperature and stored at 0°C for 24h. Recrystallisation of the solid from water afforded a yellow crystalline product which was collected by filtering under reduced pressure, washed with water and ethanol and was dried to yield (15) (0.27 g, 2.6%) as a yellow solid. m.p. 244°C (lit. (Bredereck and Föhlisch., 1962) m.p. 243-244°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.38; I.R. υmax 3450 cm^{-1}, 3200 cm^{-1}, 1670 cm^{-1}; ^1H NMR δ (D_2O) 3.25 (3 H, s, N-CH_3), 3.40 (3 H, s, N-CH_3), 4.75 (2 H, s, CH_2); m/z 210 (M^+), 180 (100%).

1,3-Dimethylpyrimidine-2,4,6-trione (16)

Propanedioic acid (22 g, 211 mmol) was added to a solution of 1,3-dimethylurea (15.9 g, 180 mmol) in acetic acid (35 ml) and heated with stirring to a temperature of 70°C. After 1 h heating, acetic anhydride (72 ml) was added dropwise to the mixture and the temperature was allowed to rise to 90°C. The reaction was stirred at this temperature for a further 6 h.

On completion of the reaction, the mixture was cooled to ambient temperature with evaporation of the solvent affording a yellow crystalline mass which was collected by filtration, washed with ethanol (18 ml) and recrystallised from ethanol to afford (16) (20.74 g, 74%) as fine yellow needles. m.p. 120-121°C (lit. (Goldner *et al.*, 1966)

m.p. 124-126°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.78; I.R. υmax 1640cm^{-1}; ^{1}H NMR δ (D_2O) 3.23 (6 H, s, $(N\text{-}CH_3)_2$), 4.80 (2 H, s, 5-H); m/z 156 (M^+), 42 (100%).

1,3-Dimethyl-6-chloropyrimidine-2,4-dione (17)

Phosphorus oxychloride (120 ml, 780 mmol) was added dropwise to a stirred suspension of 1,3-dimethylpyrimidine-2,4,6-trione (15.6 g, 100 mmol) in water (5 ml). The mixture was boiled under reflux for 40 min, after which time the excess phosphorus oxychloride was removed by distillation under reduced pressure to reveal a brown gum. Ice was added to this gum which dissolved in the aqueous solution. The aqueous solution was then extracted twice with CH_2Cl_2, the combined organic extracts were collected then dried. The solvent was evaporated yielding (17) (11.27 g, 65%) as a dark yellow solid. m.p. 109°C (lit. (Goldner *et al.*, 1966) m.p. 109-110°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.85; I.R. υmax 1719 cm^{-1}, 720 cm^{-1}; ^{1}H NMR δ ($CDCl_3$) 3.40 (3 H, s, $N\text{-}CH_3$), 3.50 (3 H, s, $N\text{-}CH_3$), 6.00 (1 H, s, 5-H); m/z 174 (M^+), 82 (100%).

1,3-Dimethyl-6-(2-hydroxyethylamino)pyrimidine-2,4-dione (18)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (3.7 g, 21 mmol), 2-amino-ethanol (2.00 g, 33 mmol) and anhydrous sodium carbonate (4.45 g, 42 mmol) was boiled under reflux with stirring for 4 h, until formation of single product as shown by TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature, the mixture was filtered. The solvent was evaporated from the clear filtrate revealing a yellow solid which on recrystallisation with ethanol afforded (18) (3.01g, 72%) as yellow needles. m.p. 170-171°C (lit. (Golner *et al.*, 1966) m.p. 180-181°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf =

0.3; I.R. υmax 3340-3240 cm^{-1}, 1650 cm^{-1}; ^1H NMR δ (D_2O) 3.33 (3 H, s, N-CH_3), 3.55 (3 H, s, N-CH_3), 3.81 (2 H, t, J 4 Hz, CH_2-N), 3.93 (2 H, t, J 4 Hz, CH_2-O); m/z 199 (M^+), 156 (100%).

1,3-Dimethyl-6-(2-hydroxyethylamino)-5-nitrosopyrimidine-2,4-dione (19)

Isoamyl nitrite (2.9 g, 24 mmol) was slowly added to suspension of 1,3-dimethyl-6-(2-hydroxyethylamino)pyrimidine-2,4-dione (2.5 g, 12.6 mmol) in ethanol (78 ml). The mixture was stirred at ambient temperature for 10 min, after which time 10 drops of hydrochloric acid were added. On stirring for a further 30 min, the suspension changed from colourless to pink and finally to a purple colour. The reaction was continued until TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) showed formation of product. After completion of reaction the product was collected by filtration and air dried. The resultant purple solid was recrystallised from ethanol to afford (19) (2.81 g, 63%) as fine purple needles. m.p. 230°C (lit. (Goldner *et al.*, 1966) m.p. 230°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.33; I.R. υmax 3430 cm^{-1}, 1679 cm^{-1}; ^1H NMR δ (D_2O) 3.33 (3 H, s, N-CH_3), 3.55 (3 H, s, N-CH_3), 3.81 (2 H, t, J 4 Hz, CH_2-N), 3.93 (2 H, t, J 4 Hz, CH_2-O).

1,3-Dimethyl-3,7-dihydro-8-hydroxymethyl-(1H)-purine-2,6-dione (15)

Butanol (25 ml) was added to 1,3-dimethyl-6-(2-hydroxyethylamino)-5-nitroso-pyrimidine-2,4-dione (1.00 g, 4.4 mmol) and the mixture boiled under reflux with stirring until decolourisation of the reaction mixture, and formation of single spot on TLC (silica gel 10 CH_2Cl_2: 1 MeOH). The resulting clear solution was then cooled to ambient temperature and the solvent removed by evaporation under reduced pressure to reveal a cream coloured solid which was recrystallised from ethanol to afford (15) (0.50 g, 54%) as fine needles. m.p. 243°C (lit. (Goldner *et al.*, 1966) m.p. 243-244°

C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.39; I.R. υmax 3450 cm^{-1}, 3200 cm^{-1}, 1650 cm^{-1}; ^1H NMR δ (D_2O) 3.19 (3 H, s, N-CH_3), 3.34 (3 H, s, N-CH_3), 4.69 (2H, s, CH_2-O); m/z 210 (M^+), 180 (100%); Anal Calcd for $C_8H_{10}N_4O_3$: C, 45.71; H, 4.80; N, 26.66. Found C, 45.75; H, 4.88; N, 26.76.

1,3-Dimethyl-3,7-dihydro-8-carboxy-(1H)-purine-2,6-dione (20)

To a stirred solution of 1,3-dimethyl-3,7-dihydro-8-hydroxymethyl-(1H)-purine-2,6-dione (0.3 g, 1.4 mmol) in water (0.7 ml), 2 M aqueous NaOH (1 ml) was added at 0° C. To this solution, 5 portions of potasium permanganate (0.32 g in 5.5 ml of water) were added dropwise and the resultant purple mixture stirred at ambient temperature for 3 h.

After 3 h, the resultant brown precipitate was removed by filtering under reduced pressure to reveal a clear filtrate which was acidified to pH 1 with concentrated hydrochloric acid and stored at 0°C for 24 h.

The yellow solid was collected by filtration, washed and recrystallised from water to yield (20) (0.17 g, 53%) as yellow needles. m.p. 269-272°C (lit. (Bredereck and Föhlisch, 1966) m.p. 273°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.54; I.R. υ max 3440 cm^{-1}, 3180 cm^{-1}, 1650 cm^{-1}; ^1H NMR δ (CF_3CO_2D) 3.63 (3 H, s, N-CH_3), 3.84 (3 H, s, N-CH_3); m/z 224 (M^+), 180 (100%); Anal calcd for $C_8H_8N_4O_4$: C, 42.86; H, 3.60; N, 24.99. Found C, 42.98; H, 3.55; N, 25.17.

1,3-Dimethyl-6-(3-hydroxypropylamino)pyrimidine-2,4-dione (21)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (5.00 g, 29 mmol), 3-amino-propanol (2.8 g, 37 mmol) and sodium carbonate (4.45 g, 42 mmol) in butanol (120 ml) was boiled under reflux with stirring until formation of single product on TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature, the mixture was filtered, the precipitate was removed and the remaining clear filtrate was evaporated to reveal a white solid which was recrystallised from ethanol to afford (21) (4.89 g, 80%) as white needles. m.p. 150°C; TLC (silica gel 10 CH_2Cl_2 : 1MeOH) Rf = 0.49; I.R υmax 3460-3420cm^{-1}, 1630cm^{-1}; ^1H NMR δ (D_2O) 1.81 (2 H, q, J 7 Hz, CH_2), 3.13 (3 H, s, N-CH_3), 3.20 (2 H, t, J 7 Hz, CH_2-N), 3.25 (3 H, s, N-CH_3), 3.64 (2 H,t, J 6 Hz, CH_2-O), 4.86 (1 H, s, 5 H); m/z 214 (M^+), 31 (100%); Anal. calcd. for $C_9H_{15}N_3O_3.1H_2O$: C, 46.70; H, 7.41; N, 18.16. Found C, 46.40; H, 7.46; N, 18.20.

1,3-Dimethyl-6-(3-hydroxypropylamino)-5-nitrosopyrimidine-2,4-dione (22)

Isoamyl nitrite (2.09 g, 18 mmol) was slowly added to a suspension of the 1,3-dimethyl-6-(3-hydroxypropylamino)pyrimidine-2,4-dione (1.8 g, 7.4 mmol) in ethanol (57 ml). The mixture was stirred at ambient temperature for 10 min, after which time 10 drops of hydrochloric acid was added. On stirring for a further 30 min, the suspension changed colour from clear to pink. The reaction was continued until TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) showed formation of the nitroso prduct.

After reaction had gone to completion, the purple product was collected by filtering under reduced pressure to afford a pink solid which was recrystallised from ethanol to give (22) (0.39 g, 20%) as fine pale pink needles. m.p. 278°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.56; I.R υmax 1640 cm^{-1}; ^1H NMR δ (D_2O) 1.95 (2 H, q, J 6 Hz, CH_2), 2.99 (2 H, t, J 6 Hz, CH_2-N), 3.33 (3 H, s, N-CH_3), 3.56 (3 H, s, N-CH_3), 3.69 (2 H, t, J 6 Hz, CH_2-O); m/z 225 (M^+), 181 (100%); Anal Calcd for $C_9H_{14}N_4O_4$: C, 44.63; H, 5.83; N, 23.13. Found C, 44.80; H, 5.76; N, 23.20.

1,3-Dimethyl-3,7-dihydro-8-(2-hydroxyethyl)-(1H)-purine-2,6-dione (23)

A mixture of 1,3-dimethyl-6-(3-hydroxypropylamino)-5-nitrosopyrimidine-2,4-dione (0.30 g, 1.2 mmol) in butanol (50 ml) was boiled under reflux with stirring for 1 h until the purple nitroso solution decolourised. The reaction was monitored by TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) for formation of single product.

After cooling to ambient temperature, the resulting clear solution was evaporated to reveal a cream coloured solid which, on recrystallisation with ethanol, afforded (23) (0.28 g, 82%) as fine white needles. m.p. 278°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.69; I.R υmax 3360 cm^{-1}, 1650 cm^{-1}; ^1H NMR δ (D_2O) 2.94 (2 H, t, J 6 Hz, CH_2), 3.17 (3 H, s, N-CH_3), 3.34 (3 H, s, N-CH_3), 3.89 (2 H, t, J 6 Hz, CH_2O); m/z 225 (M^+), 185 (100%). Anal. Calcd. for ($C_9H_{12}N_4O_3$): C, 48.21; H, 5.40; N, 24.99. Found C, 47.9; H, 5.26; N, 24.90.

1,3-Dimethyl-6-(2-methoxyethylamino)pyrimidine-2,4-dione (24)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (2.00 g, 11 mmol), 2-methoxyethylamine (1.12 g, 15 mmol) and sodium carbonate (2.33 g, 22 mmol) in butanol (65 ml) was boiled under reflux with stirring for 4 h until formation of single product on TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature the mixture was filtered, the precipitate was removed and the remaining clear filtrate was evaporated to reveal an off-white solid which was recrystallised from tetrahydrofuran to afford (24) (2.12 g, 91%) as cream needles. m.p. 190°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.55; I.R.υmax 3250 cm^{-1} 1700 cm^{-1}, 1640-1600 cm^{-1}, 1550 cm^{-1}; ^{1}HNMR δ [$(CD_3)_2SO$] 3.07 (3 H, s, OCH_3), 3.22 (2 H, q, J 6 Hz, C*H*$_2$-NH), 3.25 (3 H, s, N-CH_3), 3.46 (2 H, t, J 6 Hz, C*H*$_2$-OCH_3), 3.57 (3 H, s, N-CH_3), 4.70 (1 H, s, 5-H), 6.76 (1 H. br, NH); m/z 210

(M^+), 180 (100%). Anal Calcd for ($C_9H_{13}N_3O_3$): C, 51.18; H, 6.20; N, 19.89. Found C, 51.2; H, 6.25; N, 19.86.

1,3-Dimethyl-6-(2-methoxyethylamino)-5-nitrosopyrimidine-2,4-dione (25)

To a suspension of 1,3-dimethyl-6-(2-methoxyethylamino)pyrimidine-2,4-dione (1.5 g, 7.03 mmol) in water (7 ml), sodium nitrite ().74 g, 10.7 mmol) was added portionwise. Over a 1 h period, acetic acid (0.74 ml) was added dropwise to the suspension, after which time a colour change occurred from clear to purple. The mixture was then stirred at ambient temperature until TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) showed formation of single product.

After completion of the reaction the purple precipitate was collected by filtering under reduced pressure. It was washed with water and recrystallised from ethanol to afford (25) (1.00 g, 58%) as purple needles. m.p 232-234°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.59; I.R. υmax 3440 cm^{-1}, 3180 cm^{-1}, 1720 cm^{-1}, 1640 cm^{-1}, 1570 cm^{-1}, 1500 cm^{-1}; ^1H NMR δ (D_2O) 3.34 (3 H, s, N-CH_3), 3.46 (3 H, s, OCH_3), 3.56 (3 H, s, N-CH_3), 3.69 (2 H, t, J 6 Hz, CH_2-OCH_3), 4.00 (2 H, t, J 6 Hz, CH_2-NH); m/z 226 (M^+), 210 (100%).Anal Calcd for ($C_9H_{14}N_4O_4$): C, 44.63; H, 5.83; N, 23.13. Found C, 44.67; H, 5.80; N, 23.26.

1,3-Dimethyl-3,7-dihydro-8-methoxymethyl-(1H)-purine-2,6-dione (26)

A mixture of 1,3-dimethyl-6-(2-methoxyethylamino)-5-nitrosopyrimidine-2,4-dione (0.39 g, 1.60 mmol) in butanol (70 ml) was boiled under reflux with stirring for 3 h or until the purple nitroso solution decolourised. The reaction was monitored by TLC for formation of single product.

After cooling to ambient temperature the resulting solution was evaporated to reveal a cream solid which, on recrystallisation from ethanol, afforded (26) (0.27 g, 75%) as a

cream powder. m. p. 165°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.6; I.R. υ max 3400 cm^{-1}, 3200-3100 cm^{-1}, 1700 cm^{-1}, 1650 cm^{-1}, 1600 cm^{-1}, 1510 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 3.23 (3 H, s, N-CH_3), 3.31 (3 H, s, OCH_3), 3.43 (3 H, s, N-CH_3), 4.45 (2 H, s, CH_2-O); m/z 224 (M^+), 181 (100%); Anal Calcd. for ($C_9H_{12}N_4O_3$): C, 48.21; H, 5.39; N, 24.99. Found C, 48.19; H, 5.38; N, 25.01.

1,3-dimethyl-3,7-dihydro-8-nitropurine-2,6-dione (28)

A mixture of theophylline (20 g, 111 mmol) and glacial acetic acid (40 ml) was warmed to a temperature of 87°C. To this solution concentrated nitric acid (19.6 g, 311 mmol) was added dropwise over a period of 1.5 h, with temperature of reaction kept below 105°C.

After reaction completion, the resulting yellow mixture was cooled, the solid collected and washed several times with water, dried to afford 1,3-dimethyl-3,7-dihydro-8-nitropurine-2,6-dione (16) (17 g, 66%) as a powdery yellow solid .m.p. 280°C (lit. (Duesel, 1958) m.p. 282°C); TLC (silica gel 9 CH_2Cl_2 : 1 MeOH) Rf = 0.59; I.R υ max 3500-3400 cm^{-1}, 3250 cm^{-1}, 2980 cm^{-1}, 2600-2500 cm^{-1}, 1700 cm^{-1}, 1670-1640 cm^{-1}, 1530 cm^{-1}, 1350 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 3.25 (3 H, s, N-CH_3), 3.43 (3 H, s, N-CH_3), 8.41 (1 H, s, NH) Anal. Calcd. for ($C_7H_7N_5O_4$): C, 37.12; H, 3.13; N, 31.10. Found C, 37.25; H, 3.35; N, 31.19.

1,1-Dimethylethyl N-(2-(2-hydroxyethoxy)ethoxy)carbamate (29)

Di-tert-butyl dicarbonate (4.15 g, 19 mmol) in dichloromethane (50 ml) was added dropwise to a solution of 2-(2-aminoethoxy)ethanol (2.00 g, 19 mmol) in dichloro-methane (50 ml). The resulting solution was stirred at ambient temperature for 24 h.

The solvent was removed by evaporation to reveal (17) (3.89 g, 99%) as a pale yellow oil ; TLC (silica gel 20 CH_2Cl_2 : 1 MeOH) Rf = 0.30; I.R(film) υmax 3500-

3200 cm^{-1}, 1700 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.45 (9 H, s, $(CH_3)_3$), 2.46 (1 H, s, N-H), 3.32 (2 H, q, J 6 Hz, CH_2-N), 3.58 (4 H, m, CH_2-O-CH_2), 3.75 (2 H, t, J 6 Hz, CH_2-OH), 5.31 (1 H, s, OH); m/z 206, 106 (100%).

1,1-Dimethylethyl N-(2-(2-(2-cyanoethoxy)ethoxy)ethyl)carbamate (30)

Acrylonitrile (1.19 g, 23 mmol) was added dropwise to a stirred solution of 1,1-dimethylethyl N-(2-(2-hydroxyethoxy)ethoxy)carbamate (3.00 g, 15 mmol) containing 10 drops of benzyltrimethylammonium hydroxide. The resulting solution was then stirred at ambient temperature for 24 h.

The brown solution was diluted with dichloromethane, washed four times with water, the combined organic extracts were dried with evaporation of the solvent yielding () (2.62 g, 68%) as an orange-brown oil ; TLC (silica gel 20 CH_2Cl_2 : 1 MeOH) Rf = 0.70; I.R.(film) υmax 3380 cm^{-1}, 2260 cm^{-1}, 1740 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.45 (9 H, s, $(CH_3)_3$), 2.64 (2 H, m, CH_2-CN), 3.54 (2 H, q, J 6 Hz, CH_2-N), 3.73 (8 H, m, 2 × CH_2-O-CH_2), 5.31 (1 H, s, N-H); m/z 246, 106 (100%).

Phenylmethyl N-(2-(2-hydroxyethoxy)ethyl)carbamate (31)

Phenylmethyl chloroformate (9.74 g, 57 mmol) in dichloromethane (15 ml) was added dropwise to a solution of 2-(2-aminoethoxy)ethanol (6.00 g, 57 mmol) and triethylamine (6.35 g, 63 mmol) in dichloromethane (25 ml). The resulting solution was stirred at ambient temperature for 24 h.

The solution was quenched with water (50 ml) and the organic layer was extracted, washed twice with 10% aqueous H_2SO_4 and water. The combined organic extracts were collected and dried. Evaporation of the solvent afforded (19) (8.5 g, 62%) as a pale yellow oil; TLC (silica gel 20 CH_2Cl_2 : 1 MeOH) Rf = 0.5; I.R.(film) υmax 3440-3300 cm^{-1}, 1720 cm^{-1}, 1560 cm^{-1}; ^1H NMR δ ($CDCl_3$) 3.20 (1 H, s, N-H), 3.35 (2 H,

t, J 6 Hz, CH_2-N), 3.48 (4 H, m, $(CH_2)_2O$), 3.68 (2 H, m, CH_2-O), 5.82 (1 H, s, OH), 7.32 (5 H, s, Aryl-H); m/z 240 (M^+), 91 (100%).

Phenylmethyl N-(2-(2-(2-cyanoethoxy)ethoxy)ethyl)carbamate (32)

Acrylonitrile (0.99 g, 19 mmol) was added dropwise to a stirred solution of phenylmethyl N-(2-(2-hydroxyethoxy)ethyl)carbamate (3.00 g, 13 mmol) containing 10 drops of benzyltrimethylammonium hydroxide. The resulting brown solution was stirred at ambient temperature for 24 h.

The mixture was diluted with dichloromethane and was washed four times with water. The organic extracts were collected and dried and the solvent was removed to yield (20) (3.57 g, 98%) a brown oil; TLC (silica gel 20 CH_2Cl_2 : 1 MeOH) Rf = 0.59; I.R(film) υmax 3500-3400 cm^{-1}, 2250 cm^{-1}, 1730 cm^{-1}; ^1H NMR δ ($CDCl_3$) 3.01 (2 H, m, CH_2-CN), 3.80 (2 H, m, CH_2-N), 3.96 (8 H, m, 2 × CH_2-O-CH_2), 5.45 (1 H, br, N-H), 7.62 (5 H, s, Aryl-H).

Phenylmethyl N-(2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (35)

Phenylmethyl chloroformate (6.0 g, 35 mmol) in dichloromethane (20 ml) was added dropwise to a stirred solution of 3,6-dioxaoctane-1,8-diamine (52.1 g, 352 mmol), triethylamine (7.08g, 70 mmol) in dichloromethane (100 ml). The homogeneous mixture was left to stir at ambient temperature for 24 h.

After overnight stirring, the reaction mixture was washed once with water and the organic extract was acidified with 2M hydrochloric acid, collected then dried with evaporation of the solvent affording (36) (3.65 g, 50%) as a pale oil which crystallised on standing. The remaining aqueous extract was made basic with 2M NaOH extracted thrice with EtOAc, the organic extracts were then collected, dried with evaporation of the solvent affording (35) (0.22 g, 13.3%) as a pale oil ; TLC (silica gel 10 CH_2Cl_2 : 1

MeOH) Rf = 0.3; I.R. υmax 3250 cm^{-1}, 1720 cm^{-1}, 1640 cm^{-1}; ^1H NMR δ 2.64 (2H, m, CH_2-NH_2), 3.18 (2H, m, CH_2-NH), 3.42 (4H, m, $(CH_2$-$O)_2$), 3.52 (4H, m, CH_2CH_2-NH), 5.03 (2H, br, Aryl-CH_2), 7.36 (5H, br, Aryl); m/z 283 (M^+), 91 (100%).

2-(2-(2-phthalimidoethoxy)ethoxy)ethanol (38)

2-(2-(2-chloroethoxy)ethoxy)ethoxy (1.5 g, 8.91 mmol) was added to a mixture of potassium phthalimide (1.5 g, 8.10 mmol) in dimethylformamide (2 ml) and left to stir at 120°C for 5 h.

After completion of the reaction, the resulting precipitate was removed by filtration, with evaporation of the clear filtrate revealing a yellow-brown oil which was purified by column chromatography in the system (7 EtOAc : 7 Hexane : 1 MeOH) to afford (38) as a pale yellow oil (0.45 g, 20%); TLC (silica gel 7 EtOAc : 7 Hexane : 1 MeOH) Rf = 0.31; I.R.υmax 3480-3400 cm^{-1}, 2880 cm^{-1}, 1765 cm^{-1}, 1710 cm^{-1}, 1680 cm^{-1}, 1470 cm^{-1}, 1390 cm^{-1}, 1200 cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 2.97 (1 H, s, OH), 3.5-4.01 (12 H, m, NCH_2 + OCH_2) 7.65 (4 H, m, Aryl).

2-(2-(2-Phthalimidoethoxy)ethoxy)acetic acid (39)

To a stirred solution of the 2-(2-(2-phthalimidoethoxy)ethoxy)ethanol (1.91g, 6.8 mmol) in acetone (133 ml) Jones reagent ($K_2Cr_2O_7$ in H_2SO_4) (7.6 ml) was added dropwise. The mixture was stirred at ambient temperature until disappearance of starting material.

After 5 h stirring the reaction was complete, the resulting precipitate was removed by filtration and the clear filtrate evaporated under reduced pressure to afford a pale yellow oil. The oil was then dissolved in CH_2Cl_2, washed twice with water (10 ml), once with 2 M hydrochloric acid (15 ml), the organic extracts were collected then

dried , the solvent removed by evaporation under reduced pressure to afford a pale oil which after trituration with ether gave (39) (1.68g, 84%) as a white crystalline solid. m.p.270°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.31; I.R. υmax 3700-3340 cm^{-1}, 1770 cm^{-1}, 1710 cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 3.71 (4 H, br, $(CH_2O)_2$), 3.77 (2 H, m, CH_2), 3.93 (2 H, t, CH_2-NH), 4.10 (2 H, m, CH_2CO_2H), 5.70 (1 H, br, CO_2H), 7.74-7.87 (4 H, m, Aryl-H_4); m/z 294.1 (M^+), 27.9 (100%). Anal calcd for ($C_{14}H_{15}NO_6$): C, 57.34; H, 5.16; N, 4.78. Found C, 57.40; H, 5.21; N, 4.75.

1,3-dimethyl-6-amino-5-(2-(2-(2-phthalimidoethoxy)ethoxy)acetylamino)pyrimidine-2,4-dione (40)

To a solution of 5,6-diamino-1,3-dimethyluracil (0.34 g, 2 mmol) in dimethylformamide (8 ml), dicyclohexylcarbodiimide (0.41 g, 2 mmol) and phthalimido acid (0.59 g, 2 mmol) was added portionwise. The solution was allowed to stir at ambient temperature for 24 h with a further addition of dimethylformamide (4 ml).

The resulting precipitate was removed by filtration, the solvent evaporated under reduced pressure to afford an orange oil which was dissolved in dichloromethane, washed twice with water, the organic extracts collected, dried to reveal a pale yellow solid (1.05 g). The product was purified by column chromatography in the eluting system (9 CH_2Cl_2 : 1 MeOH), collected with evaporation of the solvent revealing a pale yellow solid which when trituated with EtOAc resulted in (40) (0.11 g, 12%) as a creamy colourless solid. m.p. 230°C; TLC (silica gel 9 CH_2Cl_2 : 1 MeOH) Rf = 0.72; I.R.υ max 3400 cm^{-1}, 3360 cm^{-1}, 1720 cm^{-1}, 1650cm^{-1}; ^{1}H NMR δ [$(CD_3)_2SO$] 3.05 (3 H, s, N-CH_3), 3.26 (3 H, s, N-CH_3), 3.61 (2 H, m, phth-CH_2), 3.72 (2 H, q, CH_2-O), 3.99 (4H, m, $(CH_2$-O$)_2$), 6.61 (1 H, br, 5-NH), 7.86 (4 H, m, Aryl); m/z 445

(M^+); Anal Calcd for ($C_{20}H_{23}N_5O_7$): C, 54.05; H, 4.99; N, 15.76. Found C, 54.10; H, 5.21; N, 15.75.

Phenylmethyl N-[6-(1,1-dimethylethoxycarbonylamino)hexyl]carbamate (42)

Phenylmethyl chloroformate (0.61 g, 3.56 mmol) in dichloromethane (6 ml) was added dropwise, over a 30 min period, to a stirred solution of 1,1-dimethylethyl N-(6-aminohexyl)carbamate hydrochloride (0.90 g, 3.56 mmol) and triethylamine (0.72 g, 7.12 mmol) in dichloromethane (12 ml). The mixture was left to stir at ambient temperature for 24 h.

The resulting precipitate was removed by filtration, the clear filtrate was washed twice with water and once with 2M hydrochloric acid. The organic extracts were collected and dried, with removal of solvent, affording (42) (0.96 g, 77%) as a white crystalline solid. m.p. 190°C ; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.62; I.R υmax 3340 cm^{-1}, 1680 cm^{-1}, 1550 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.44 (17 H, m, $(CH_3)_3$ + $(CH_2)_4$), 1.85 (1 H, br, N-H), 3.11 (2 H, q, J 6 Hz, C*H*$_2$NH), 3.17 (2 H, m, C*H*$_2$NH), 4.85 (1 H, br, N-H), 5.09 (2 H, s, Aryl-CH_2), 7.35 (5 H, s, Aryl); m/z 350 (M^+); Anal Calcd for $C_{19}H_{30}N_2O_4$; C, 65.12; H, 8.63; N, 7.99. Found C, 65.41; H, 8.61; N, 8.17.

Phenylmethyl N-(6-aminohexyl)carbamate hydrochloride(43)

Hydrogen chloride was bubbled through a solution of phenylmethyl N-[6-(1,1-dimethylethoxycarbonylamino)hexyl]carbamate (0.96 g, 2.7 mmol) in CH_2Cl_2 (100 ml) until precipitation of the desired product. The solvent was evaporated to afford (43) (0.68 g, 99%) as a white crystalline salt. m.p. 177°C (lit. (Atwell and Denny, 1977) m.p. 177-178°C); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.1; I.R υmax 3470 cm^{-1}, 3340-3280 cm^{-1}, 2750 cm^{-1}, 2620 cm^{-1}, 2500 cm^{-1}, 1700 cm^{-1}, 1630 cm^{-1}, 1570 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.18 (4 H, m, 2 × CH_2), 1. (4 H, m, 2 × CH_2), 2.93 (2 H, t, J 6

Hz, $CH_2NH_3^+$), 3.08 (2 H, m, J 6 Hz, CH_2NH), 3.61 (3 H, N^+H_3), 5.07 (2 H, s, Aryl-CH_2), 7.39 (5 H, s, Aryl); m/z 287 (M^+); Anal Calcd for ($C_{14}H_{22}N_2O_2$.HCl); C, 58.63; H, 8.08; N, 9.77. Found C, 58.65; H, 8.03; N, 9.74.

1,3-Dimethyl-6-[6-(phenylmethoxycarbonylamino)hexylamino]pyrimidine-2,4-dione (44)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (0.49 g, 2.85 mmol), phenylmethyl N-(6aminohexyl)carbamate (840 mg, 3.7 mmol) and sodium carbonate (0.60 g, 5.7 mmol) in dioxane (8 ml) was boiled under reflux with stirring for 4 h until TLC (silica gel 10 CH_2Cl_2 :.1 MeOH) showed formation of single product.

After cooling the mixture to ambient temperature chloroform was added. The solution was washed twice with water, once with saturated brine then dried. Evaporation of the solvent revealed a yellow semi-oily solid which on further standing solidified to a yellow solid which was recrystallised from ethanol to afford (44) (0.60 g, 54%) as a yellow crystalline solid. m.p. 128°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.55; I.R.υmax 3360 cm^{-1}, 3280 cm^{-1}, 1700 cm^{-1}, 1630 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 0.90 (4 H, m, 2 × CH_2), 1.23 (4 H, m, 2 × CH_2), 3.15 (2 H, t, J 6 Hz, $NHCH_2$), 3.17 (2 H, t, J 6 Hz, $NHCH_2$), 3.06 (3 H, s, N-CH_3), 3.23 (3 H, s, N-CH_3), 4.21 (1 H, dt, pyrimidine-5H + NH), 4.69 (2 H, $PhCH_2$), 6.79 (5 H, m, Ph-H_5), 7.70 (1H, br, NH); m/z 388 (M^+) and 91 (100%); Anal Calcd for ($C_{20}H_{28}N_4O_4$): C, 61.82; H, 7.27; N, 14.43. Found C, 61.85; H, 7.36; N, 14.3.

1,3-dimethyl-6-[6(phenylmethoxycarbonylamino)hexylamino]-5-nitrosopyrimidine-2,4-dione (45)

Isoamyl nitrite (0.41 g, 3.54 mmol) was slowly added to a warmed solution of 1,3-dimethyl-6-[6-(phenylmethoxycarbonlyamino)hexylamino]pyrimidine-2,4-dione

(0.55 g, 1.42 mmol) in ethanol (25 ml). The mixture was stirred at ambient temperature for 10 min after which time 10 drops hydrochloric acid was added, the reaction was stirred at this temperature until TLC (silica gel 10 CH_2Cl_2: 1 MeOH) showed formation of nitroso product.

After overnight stirring at ambient temperature the resulting yellow solution was evaporated under reduced pressure to afford (45) a red oil (0.43 g, 73%). This product was subsequently used without further purification in the next step.

1,3-dimethyl-3,7-dihydro-8-phenylmethoxycarbonylpentaethyl-2,6-dione (46)

A solution of 1,3-dimethyl-6-[6(phenylmethoxycarbonylamino)hexylamino]-5-nitroso-pyrimidine-2,4-dione (0.43 g, 1.03 mmol) in butanol (3 ml) was boiled under reflux with stirring until decolourisation of solution had occurred.

After cooling the solution to ambient temperature, the solvent was removed to afford a pale grey solid which was washed with ether, collected and recystallised fom butanol to reveal (46) (0.40 g, 97%) as a grey crystalline solid. m.p. 182-184°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.65; I.R.υmax 3300 cm^{-1}, 1700 cm^{-1}, 1680 cm^{-1}, 1660 cm^{-1}, 1550 cm^{-1}, 1500 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.55 (2 H, quintet, J 7.0 Hz, CH_2), 1.67 (2 H, sextet, J 7.0 Hz, CH_2), 1.90 (2H, quintet, J 7.3 Hz, CH_2), 1.92 (2 H, t, J 7.3 Hz, purine-CH_2), 3.23 (2 H, q, J 6.4 Hz, CH_2-NH), 3.45 (3 H, s, N-CH_3), 3.59 (3 H, s, N-CH_3), 5.23 (2 H, br, Aryl-CH_2), 7.54 (1 H, br, Aryl-NH), 7.58 (5 H, br, Aryl); m/z 400 (M^+), 292 (M-$PhCH_2OH$) and 91 (100%); Anal Calcd. for ($C_{20}H_{25}N_5O_4$): C, 60.14; H, 6.31; N, 17.53. Found C, 60.09; H, 6.34; N, 17.50.

1,3-dimethyl-6-[6-(phenylmethoxycarbonylamino)hexylamino]-pyrimidine-2,4-dione (53)

6-Chloro-1,3-dimethylpyrimidine-2,4-dione (3.61 g, 41 mmol) was boiled under reflux with hexane-1,6-diamine (6.0 g, 52 mmol) and sodium carbonate (4.38 g, 41 mmol) in dioxane (90 ml) for 3.5 h. The mixture was diluted with chloroform, washed twice with water and dried. Evaporation of the solvent gave (47) as a crude product (5.27g). This material was stirred with phenylmethyl chloroformate (3.76 g, 22 mmol) and triethylamine (4.19 g, 41 mmol) in dichloromethane (180 ml) for 24 h. After overnight stirring, the resulting precipitate was removed by filtration, the filtrate washed twice with water, once with 2M aqueous HCl and dried. Evaporation of the solvent afforded the crude product a pale cream solid which was chromatographed on silica in the system chloroform : EtOAc (4 : 1) with elution of product with EtOAc yielding the Cbz-aminoalkylaminopyrimidine (53) (4.25 g, 52%) as a white crystalline solid. m.p. 128-129°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.59; I.R.υmax 3360cm^{-1}, 3280cm^{-1}, 1700cm^{-1}, 1630cm^{-1}, 1600cm^{-1}, 1550cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 1.36 (4 H, m, 2 × CH_2), 1.52 (2 H, quintet, J 6.5 Hz, CH_2), 1.64 (2 H, quintet, J 6.9 Hz, CH_2), 3.05 (2 H, dt, J 5.3 Hz and 6.0 Hz, $NHCH_2$), 3.08 (2 H, dt, J 6.4 Hz and 6.8 Hz, $NHCH_2$), 3.31 (3 H, s, N-CH_3), 3.40 (3 H, s, N-CH_3), 4.82 (1 H, br, N-H), 4.83 (1 H, s, pyrimidine-5H), 4.88 (1 H, br, NH), 5.08 (2 H, s, $PhCH_2$), 7.34 (5 H, m, Ph-H_5); m/z 388 (M^+) and 91 (100%); Anal Calcd for ($C_{20}H_{28}N_4O_4$): C, 61.82; H, 7.27; N, 14.43. Found C, 61.5; H, 7.34; N, 14.4.

1,3-Dimethyl-6-[9-(phenylmethoxycarbonylamino)nonylamino]pyrimidine-2,4-dione (54)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (1.91 g, 11 mmol), nonane-1,9-diamine (5.00 g, 32 mmol) and sodium carbonate (2.79 g, 16 mmol) in dioxane

(60 ml) was boiled under reflux with stirring for 3 h or until formation of single product on TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature, chloroform was added to the mixture and the solution washed once with water, collected and dried. Evaporation of solvent gave the crude amine 1,3-dimethyl-6-(9-aminononylamino)pyrimidine-2,4-dione (48) (3.16 g) as a pale yellow solid. This crude material (3.16 g, 11 mmol) was then stirred with phenylmethyl chloroformate (1.91 g, 2.11 mmol) and triethylamine (2.16 g, 21 mmol) in dichloromethane (150 ml). The reaction was left to stir at ambient temperature for 24 h.

The resulting precipitate was removed by filtration, the filtrate washed twice with water, once with 2M aqueous HCl, collected and dried with evaporation of the solvent a yellow solid (3.56 g,) which was purified by flash column chromatography in the system (4 $CHCl_3$: 1 EtOAc) and flushed with EtOAc to afford (54) (2.02 g, 43%) as a white crystalline solid. m.p. 105°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.55; I.R. υmax 3420 cm^{-1}, 1700 cm^{-1}, 1680 cm^{-1}, 1630 cm^{-1}, 1600 cm^{-1}, 1550-1530 cm^{-1}; ^1H NMR δ ($CDCl_3$) 1.30 (4 H, br, 2 × CH_2), 1.50 (2 H, t, J 7.3 Hz, CH_2), 1.66 (8 H, m, $CH_2CH_2CH_2CH_2$), 3.09 (2 H, q, J 7.3 Hz, CH_2-NH), 3.18 (2 H, q, J 6.3 Hz, CH_2-NH), 3.31 (3 H, s, N-CH_3), 3.39 (3 H, s, N-CH_3), 4.35 (1 H, br, NH), 4.79 (1 H, br, Aryl-NH), 4.86 (1 H, s, 5H), 5.09 (2 H, s, Aryl-CH_2), 7.35 (5 H, s, Aryl); m/z 431 (M^+), 336 (M-$PhCH_2OH$) 91 (100%). Anal. Calcd. for $C_{20}H_{28}N_4O_4$: C, 61.84; H, 7.27; N, 14.42. Found C, 61.9; H, 7.35; N, 14.39.

1,3-Dimethyl-6-[10-(phenylmethoxycarbonylamino)decylamino]pyrimidine-2,4-dione (55)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (1.35 g, 7.73 mmol), decane-1,10-diamine (1.60 g, 9.28 mmol) and sodium carbonate (1.97 g, 190 mmol) in dioxan

(15 ml) was boiled under reflux with stirring for 3 h or until formation of single product on TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature, the solution was diluted with chloroform washed once with water then dried. Evaporation of solvent gave the crude amine (49) (1.24g). This material (0.80 g, 2.58 mmol) was stirred with phenylmethyl chloroformate (0.44 g, 2.58 mmol) and triethylamine (0.52 g, 5.51 mmol) in dichloromethane (120 ml). The homogeneous solution was then stirred at ambient temperature until formation of single product on TLC (silica gel 10CH_2Cl_2 : 1 MeOH).

After overnight stirring the resulting precipitate was removed by filtration and the solution was washed twice with water, once with 2M aqueous HCl, dried with evaporation of the solvent affording (55) as a crude product which was chromatographed on silica as for product (54) to reveal (55) (0.62 g, 54%) as a white crystalline solid; m.p. 100-102°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.56; I.R. υmax 3360 cm^{-1}, 3280cm^{-1}, 1700 cm^{-1}, 1640 cm^{-1}; ^{1}HNMR δ ($CDCl_3$) 1.29 (12 H, m, 6 × CH_2), 1.49 (2 H, m, CH_2), 1.65 (2 H, quintet, J 6.8 Hz, CH_2), 3.08 (2 H, dt, J 5.1Hz and 7.1 Hz, CH_2), 3.18 (2 H, q, J 6.6Hz, N-CH_2), 3.31 (3 H, s, N-CH_3), 3.39 (3H, s, N-CH_3), 4.40 (1 H, br, NH), 4.81 (1 H, s, pyrimidine-5H), 5.09 (2 H, s, $PhCH_2$), 7.35 (5 H, m, Ph-H_5); m/z 444 (M^+), 336 (M-$PhCH_2OH$), and 91 (100%); Anal Calcd for ($C_{24}H_{36}N_4O_4$): C, 64.82; H, 8.17; N, 12.61. Found C, 65.1; H, 8.14; N, 12.65.

1,3-Dimethyl-6-(2-{2-[2'-(phenylmethoxycarbonylamino)ethoxy]ethoxy}ethylamino)-pyrimidine-2,4-dione (56)

A mixture of 1,3-dimethyl-6-chloropyrimidine-2,4-dione (2.82 g, 16 mmol), 3,6-dioxooctane-1,8-diamine (6g, 40 mmol) and sodium carbonate (3.43 g, 32 mmol) in

dioxan (65 ml) was boiled under reflux with stirring for 3 h or until formation of single product on TLC (silica gel 10 CH_2Cl_2 : 1 MeOH).

After cooling to ambient temperature, chloroform was added to the mixture and the solution washed once with water, collected, dried with evaporation of solvent affording the crude amine (50) (3.03g). The crude 1,3-dimethyl-6-(2-{2-[2-aminoethoxy]ethoxy}ethylamino)pyrimidine-2,4-dione (3.03 g, 11 mmol) was then treated with phenylmethyl chloroformate (1.90 g, 11 mmol) and triethylamine (2.14 g, 21 mmol) in dichloromethane (150 ml). The reaction was left to stir at ambient temperature for 24 h.

The organic extract was washed twice with water, once with 2M hydrochloric acid, collected and dried. The solvent was evaporated to yield (56) as a yellow/brown oil (3.56 g) which was purified by flash column chromatography in EtOAc then 5 EtOAc : 1 MeOH affording [42] as an oil (2.04 g, 44%); TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.65; I.R. υmax 3400-3280 cm^{-1}, 1740-1670cm^{-1}; ^1H NMR δ ($CDCl_3$) 3.24 (2 H, q, J 6 Hz, $NHCH_2$), 3.31 (3 H, s, N-CH_3), 3.36 (3 H, s, N-CH_3), 3.40 (2 H, q, J 5.3 Hz, $NHCH_2$), 3.58 (2 H, t, J 6 Hz, $NHCH_2CH_2$), 3.62 (4 H, s, OCH_2CH_2O), 3.68 (2 H, t, J 6 Hz, $NHCH_2CH_2$), 4.83 (1 H, s, pyrimidine-5H), 5.00 (1 H, br, NH), 5.09 (2 H, s, Aryl-CH_2), 5.29 (1 H, br, NH), 7.35 (5 H, s, Aryl-H_5); m/z 421 (M^+), 91 (100%). Anal. Calcd for ($C_{20}H_{28}N_4O_6$):C, 57.12; H, 6.72; N, 13.33. Found C, 56.95; H, 6.78; N, 13.39.

1,3-Dimethyl-6-(9-aminononylamino)pyrimidine-2,4-dione (51)

6-Chloro-1,3-dimethylpyrimidine-2,4-dione (1.00 g, 5.7 mmol) was boiled under reflux with nonane-1,9-diamine (1.81 g, 11.4 mmol) and sodium carbonate (1.21 g, 1.4 mmol) in ethanol (40 ml) for 3 h. The mixture was filtered and the solvent was evaporated under reduced pressure to afford a pale yellow solid which was

recrystallised in aqueous ethanol to yield 6-(9-aminononylamino)-1,3-dimethyl-pyrimidine-2,4-dione (54) (1.09 g, 65%) as a white solid m.p. 210-212°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.12; I.R .υmax 3500cm^{-1}, 1660cm^{-1}; ^{1}HNMR δ [$(CD_3)_2SO$] 1.29 (14 H, m, 7 x CH_2), 2.54 (2 H, m, CH_2NH_2), 3.03 (2 H, m, $NHCH_2$), 3.03 (3 H, s, N-CH_3), 3.26 (3 H, s, N-CH_3), 4.65 (1 H, br, NH), 6.72 (1 H, s, pyrimidine 5 H); m/z 297 (M^+), 159 (100%).

1,3-Dimethyl-6-(10-aminodecylamino)pyrimidine-2,4-dione (52)

6-Chloro-1,3-dimethylpyrimidine-2,4-dione (2.00 g, 11.5 mmol) was boiled under reflux with decane-1,10-diamine (3.96 g, 23 mmol) and sodium carbonate (2.44 g, 23 mmol) in ethanol (100 ml) for 3 h or until the formation of single product on TLC (silica gel 10 CH_2Cl : 1 MeOH). After cooling to ambient temperature the mixture was filtered and the solvent was evaporated under reduced pressure to afford a white waxy-solid which was recrystallised in aqueous ethanol to yield 6-(10-amino-decylamino)-1,3-dimethylpyrimidine-2,4-dione (55) (2.30 g, 64%) as white solid; m.p.72-74°C; TLC (silica gel 10 CH_2Cl_2 ; 1 MeOH) Rf = 0.15; I.R. υmax 3400 cm^{-1}, 1660 cm^{-1}; ^{1}HNMR δ (CF_3CO_2D) 1.41 (16H, m, $(CH_2)_8$), 1.82 (2H, m, CH_2-NH_2), 3.28 (2H, s, CH_2-NH), 3.60 (3H, s, N-CH_3), 3.69 (3H, s, N-CH_3).

1,3-dimethyl-6-[6-(phenylmethoxycarbonylamino)hexylamino]-5-nitrosopyrimidine-2,4-dione (57)

Isoamyl nitrite (1.11 g, 9.51 mmol) was slowly added to a warmed solution of 1,3-di-methyl-6-[6-(phenylmethoxycarbonylamino)hexylamino]pyrimidine-2,4-dione (1.68 g, 4.32 mmol) in ethanol (33 ml). The mixture was stirred at ambient temperature for 10 min, after which time 10 drops of hydrochloric acid was added, the reaction was

monitored until TLC (silica gel 10 CH_2Cl_2: 1 MeOH) showed formation of nitroso product.

After overnight stirring, the resulting yellow solution was evaporated under reduced pressure to afford (57) a purple oil (1.07 g, 59%). This product was used without further purification in the next step.

1,3-Dimethyl-6-(9-(phenylmethoxycarbonylamino)nonylamino)-5-nitrosopyrimidine-2,4-dione (58)

Isoamyl nitrite (0.94 g, 8.03 mmol) was slowly added to a suspension 1,3-dimethyl-6-(9-(phenylmethoxycarbonylamino)nonylamino)pyrimidine-2,4-dione (1.57 g, 3.65 mmol) in ethanol (27 ml). The mixture was stirred at ambient temperature for 10 min, after which time two drops of hydrochloric acid was added and the mixture continued to stir for a further 30 min.

After reaction had gone to completion, the solvent was removed by evaporation to afford a purple oil (1.16 g, 69%) which was used in the next step without further purification

1,3-Dimethyl-6-(10-(phenylmethoxycarbonylamino)decylamino)-5-nitrosopyrimidine-2,4-dione (59)

Isoamyl nitrite (0.30 g, 2.53 mmol) was added to a suspension of 1,3-dimethyl-6-(10-(phenylmethoxycarbonylamino)decylamino)pyrimidine-2,4-dione (0.45 g, 1.01 mmol) in ethanol (20 ml). The mixture was stirred at ambient temperature for 10 min after which time a drop of HCl was added and the mixture continued stirring for a further 30 min by which time the solution changed from a clear to a pink colour.

After reaction had gone to completion, the solvent was removed under reduced pressure to afford a reddish-purple oil (0.69 g) which was used without further purification for the next step.

1,3-Dimethyl-6-(2-{2-[2'-(phenylmethoxycarbonylamino)ethoxy]ethoxy}ethylamino)-5-nitrosopyrimidine-2,4-dione (60)

Isoamyl nitrite (0.80 g, 6.84 mmol) was slowly added to a suspension of 1,3-dimethyl-6-(2-{2-[2'-(phenylmethoxycarbonylamino)ethoxy]ethoxy}ethylamino)pyrimidine-2,4-dione (1.75 g, 4.16 mmol) in ethanol (32 ml). The mixture was stirred at ambient temperature for 10 min after which time two drops of 3 M aqueous HCl was added. The mixture was continued to stir for a further 30 min by which time the solution changed from a clear to a pink colour.

After 2 h stirring, the solvent was removed by evaporation to afford (60) (1.82 g, 97%) as a reddish-purple oil which was used in the next step without further purification.

1,3-Dimethyl-3,7-dihydro-8-(5-(phenylmethoxycarbonylamino)pentyl)purine-2,6-dione (61)

A solution of 1,3-dimethyl-6-[6-(phenylmethoxycarbonylamino)hexylamino]-5-nitrosopyrimidine-2,4-dione (1.07 g, 2.56 mmol) in butanol (41 ml) was boiled under reflux with stirring until decolourisation of solution had occurred.

After 0.5 h stirring the solution was cooled to ambient temperature with evaporation of solvent affording a pale solid which was recystallised fom butanol to reveal (61) (0.88 g, 86%) as a pale buff solid m.p. 182-184°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.54; I.R.υmax 3300 cm^{-1}, 1700 cm^{-1}, 1685 cm^{-1}, 1660 cm^{-1}; ^{1}HNMR δ [$(CD_3)_2SO$] 1.31 (2 H, m, $CH_2CH_2\mathit{CH_2}CH_2CH_2$), 1.43 (2 H, m, $NCH_2\mathit{CH_2}$), 1.66

(2 H, m, purineCH_2CH_2), 2.69 (2 H, t, J 7.4Hz, purine-CH_2), 2.98 (2 H, q, J 7 Hz, NCH_2) 3.21 (3 H, s, N-CH_3), 3.37 (3 H, s, N-CH_3), 4.99 (2 H, s, Aryl-CH_2), 7.30 (1 H, t, J 7 Hz, N*H*CH_2), 7.34 (5H, m, Aryl), 13.2 (1H, br, purine-NH); m/z 400 (M^+), 292 (M-$PhCH_2OH$) and 91 (100%); Anal Calcd. for ($C_{20}H_{25}N_5O_4$): C, 60.14; H, 6.31; N, 17.53. Found C, 60.09; H, 6.34; N, 17.50.

1,3-Dimethyl-3,7-dihydro-8-(8-(phenylmethoxycarbonylamino)octyl)purine-2,6-dione (62)

A solution of the nitroso product (44) (0.84 g, 1.83 mmol) in butanol (30 ml) was boiled under reflux until decolourisation of the solution had occurred or until TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) had shown formation of cyclised product.

After 0.5h stirring the solution was cooled to ambient temperature, with evaporation of the solvent affording a brown solid which was recrystallised from butanol to reveal (62) as a cream solid (0.30 g, 37%) m.p. 145°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.66; I.R υmax 3320 cm^{-1}, 1740 cm^{-1}, 1680 cm^{-1}, 1640cm^{-}; ^1H NMR δ [$(CD_3)_2SO$] 1.24 (8 H, m, 4 × CH_2), 1.38 (2 H, t, J 6 Hz, CH_2), 1.65 (2 H, t, J 6 Hz, CH_2), 2.65 (2 H, t, J 6.2 Hz, CH_2NH), 2.95 (2 H, q, J 6.2 Hz, CH_2NH), 3.21 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 4.99 (2 H, s, Aryl-CH_2), 7.22 (1 H, br, Aryl-NH), 7.33 (5 H, m, Aryl), 13.15 (1 H, s, NH); m/z 442 (M^+), 334 (M-$PhCH_2OH$), 91 (100%); Anal. Calcd. for ($C_{23}H_{31}N_5O_4$): C, 62.57; H, 7.08; N, 15.86. Found C, 62.99; H, 7.35; N, 15.79.

1,3-Dimethyl-3,7-dihydro-8-(9-(phenylmethoxycarbonylamino)nonyl)purine-2,6-dione (63)

A solution of 1,3-dimethyl-6-(10-phenylmethoxycarbonylamino)decylamino)-5-nitrosopyrimidine-2,4-dione (0.69 g, 1.46 mmol) in butanol (15 ml) was boiled under reflux with stirring until the decolourisation of solution had occurred and TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) showed consumption of nitroso product.

After 40 min stirring, the solvent was removed by evaporation to afford a brown solid which was recrystallised from butanol to reveal (63) (0.30 g, 45%) as a brown crystalline mass. m.p. 95°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.60; I.R. υ max 3300cm^{-1}, 1730cm^{-1}, 1700cm^{-1}, 1650cm^{-1}, 1550cm^{-1}, 1500cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.24 (10 H, m, 5 × CH_2), 1.38 (2 H, t, J 6 Hz, CH_2), 1.67 (4 H, t, 2 × CH_2), 2.66 (2 H, q, J 7.6 Hz, CH_2-NH), 2.97 (2 H, q, J 6.4 Hz, purine-CH_2), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 4.99 (2 H, s, Aryl-CH_2), 7.22 (1 H, t, N-H), 7.34 (5H, m, Aryl); m/z 455 (M^+), 347 (M-$PhCH_2OH$) and 194 (100%); Anal Calcd for ($C_{24}H_{33}N_5O_4$): C, 63.26; H, 7.31; N, 15.38. Found C, 63.6; H, 7.28; N, 15.4.

1,3-dimethyl-3,7-dihydro-8-N-(2-{2-[2-(N-phenylmethoxycarbonylamino)ethoxy]-ethoxy}ethylamino)purine-2,6-dione(64)

A solution of the nitroso product (49) (1.82 g, 4.05 mmol) in butanol (67 ml) was boiled under reflux until decolourisation of the solution or TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) had shown formation of cyclised product.

After 0.5 h stirring the solution was cooled to ambient temperature, the solvent was evaporated to afford (64) (1.19 g, 68%) as a yellow oil. TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.48; I.R (film) υmax 3420 cm^{-1}, 3250 cm^{-1}, 3200 cm^{-1}, 1740 cm^{-1}, 1680 cm^{-1}, 1600 cm^{-1}, 1500 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 3.15 (2 H, q, J 5.9 Hz, NCH_2), 3.23 (3 H, s, N-CH_3), 3.40 (2 H, m, NCH_2CH_2), 3.42 (3 H, s, N-CH_3), 3.55 (2 H, m)

and 3.59 (2 H, m) (OCH_2CH_2O), 4.53 (2 H, s, purine-CH_2), 5.00 (2 H, s, Aryl-CH_2), 7.33 (6 H, m, Aryl-H_5 + NH), 13.57 (1 H, br, purine-NH); m/z 431 (M^+), 91 (100%). Anal. Calcd. for ($C_{20}H_{25}N_5O_6$): C, 55.68; H, 5.84; N, 16.23. Found C, 55.40; H, 5.72; N, 16.39.

8-(5-Aminopentyl)-2,4-dimethylpurine-1,3-dione hydrobromide (65)

To 1,3-Dimethyl-3,7-dihydro-8-(5-(phenylmethoxycarbonylamino)pentyl)purine-2,6-dione (0.23 g, 0.58 mmol) hydogen bromide in acetic acid (0.8 ml) was added. The mixture stirred at ambient temperature for 15 min. After completion of the reaction, the mixture was triturated with ether, collected by filtration, washed with a further five portions of ether, dried (P_2O_5/NaOH) to give the hydrobromide (65) (0.20 g, 100%) as a light purple solid; m.p. 250°C; TLC (silica gel 10 CH_2Cl_2 ; 1 MeOH) Rf = 0.1; I.R. υmax 3500-3400 cm^{-1}, 3240 cm^{-1}, 2980 cm^{-1}, 2860 cm^{-1}, 1740 cm^{-1}, 1680 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.31 (2 H, quintet, J 6.7 Hz, CH_2), 1.56 (2 H, quintet, J 7.3 Hz, CH_2), 1.70 (2 H, q, J 7.3 Hz, CH_2), 2.70 (2 H, t, J 7.7 Hz, purine-CH_2), 2.75 (2 H, q, J 7.7 Hz, CH_2-NH_3), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 7.74 (1 H, br, N^+H_3); m/z 346 (M^+), 180 (100%); Anal Calcd for $C_{12}H_{20}N_5O_2Br$: C, 41.63; H, 5.82; N, 20.23. Found C, 41.57; H, 5.77; N, 20.22.

8-(8-Aminooctyl)-2,4-dimethylpurine-1,3-dione hydrobromide (66)

To 1,3-Dimethyl-3,7-dihydro-8-(8-(phenylmethoxycarbonylamino)octyl)purine-2,6-dione (0.3 g, 0.68 mmol) hydrogen bromide in acetic acid (1.0 ml) was added. The mixture was stirred at ambient temperature for 15 min. After completion of the reaction, the mixture was triturated with ether, collected by filtration and washed with a further five portions of ether, dried to give 8-(8-aminooctyl)-2,4-dimethylpurine-1,3-dione hydrobromide (66) (0.26 g, 100%); m.p. 239°C; TLC(silica

gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.15; I.R. υmax 3440 cm^{-1}, 3200 cm^{-1}, 2940 cm^{-1}, 2860 cm^{-1}, 1760 cm^{-1}, 1680 cm^{-1}, 1570 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.27 (8 H, br, $(CH_2)_4$), 1.52 (2 H, q, CH_2), 1.67 (2 H, q, J 6.7 Hz, CH_2), 2.68 (2 H, t, J 7.6 Hz, purine-CH_2), 2.76 (2 H, sextet, J 6.4 Hz, CH_2-N^+H_3), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 7.72 (1 H, br, N^+H_3); m/z 388 (M^+), 180 (100%). Anal calcd for $C_{15}H_{26}N_5O_2Br$: C, 46.42; H, 6.75; N, 18.04. Found C, 46.41; H, 6.67; N, 18.10.

8-(9-Aminononyl)-2,4-dimethylpurine-1,3-dione hydrobromide (67)

To 1,3-Dimethyl-3,7-dihydro-8-(9-(phenylmethoxycarbonylamino)nonyl)purine-2,6-dione (0.30 g, 0.657 mmol) hydrogen bromide in acetic acid (1.0 ml) was added and the mixture stirred at ambient temperature for 15 min. After completion of the reaction, the mixture was triturated with ether, collected by filtration and washed with a further five portions of ether, dried to give 8-(9-aminonyl)-2,4-dimethylpurine-1,3-dione hydrobromide (67) (0.23 g, 87%) as a purple-grey crystalline solid; m.p. 218-219°C; TLC(silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.1; I.R. υmax 3160 cm^{-1}, 1720 cm^{-1}, 1680 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.25 (10 H, br, $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2$), 1.51 (2 H, m, purine-$CH_2$), 1.67 (2 H, m, $NHCH_2CH_2$), 2.68 (2 H, t, J 7.5 Hz, purine-CH_2), 2.75 (2 H, m, NCH_2), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 7.71 (3 H, s, N^+H_3); m/z 403 (M^+), 180 (100%). Anal calcd for $C_{16}H_{28}N_5O_2Br$: C, 57.71; H, 4.41; N, 12.24. Found C, 57.84; H, 4.47; N, 12.22.

8-{2-[2-(2-Aminoethoxy)ethoxy]ethyl}-2,4-dimethylpurine-1,3-dione hydrobromide (68)

To 1,3-dimethyl-3,7-dihydro-8-N-(2-{2-[2-(N-phenylmethoxycarbonylamino)-ethoxy]ethoxy}ethylamino)purine-2,6-dione (0.30 g, 0.70 mmol) hydrogen bromide in

acetic acid (1.0 ml) was added and the mixture stirred at ambient temperature for 15 min. After completion of the reaction, the mixture was triturated with ether, collected by filtration and washed with a further five portions of ethyl acetate (distilled and dried), dried to give the hydrobromide salt (68) (0.26 g, 100%) as a yellow solid. m.p. 220°C; TLC (silica gel 10 CH_2Cl_2 : 1 MeOH) Rf = 0.1; ^1H NMR δ [$(CD_3)_2SO$] 2.97 (2 H, sextet, J 6 Hz, CH_2N), 3.23 (3 H, s, N-CH_3), 3.43 (3 H, s, N-CH_3), 3.62 (6 H, m, $OCH_2CH_2OCH_2CH_2N$), 4.55 (2 H, s, purine-CH_2), 7.83 (3 H, br, N^+H_3); m/z 378 (M^+), 180 (100%). Anal Calcd for $C_{12}H_{20}N_5O_4Br$: C, 38.11; H, 5.33; N, 18.52. Found C, 38.15; H, 5.27; N, 18.46

5-{N-[5-(2,4-Dimethyl-1,3-dioxopurin-8-yl)pentyl]thioureido}-3',6'-dihroxyspiro(iso-benzofuran-1[3H],9'-[9H]xanthen)-3-one (69)

3'-6'-Dihydroxy-5-isothiocyanatospiro(isobenzofuran-1[3H],9'-[9H]xanthen-3-one (fluoresecein-5-isothiocyanate) (0.34 g, 0.87 mmol) was suspended in water (12 ml) and brought to pH 9.0 by dropwise addition of 1 M aqueous K_2CO_3. To this stirred solution the 8-(5-Aminopentyl)-2,4-dimethylpurine-1,3-dione hydrobromide (0.30 g, 0.87 mmol) in 2:1 H_2O/dioxan (10 ml) was added over a 30 min period. The reaction was stirred at ambient temperature until the disappearence of starting amine with the pH being carefully maintained at 9.0 by dropwise addition of 1 M aqueous K_2CO_3 throughout the course of the reaction.

After 3 h stirring at ambient temperature, the solution was acidified to pH 6.0 by dropwise addition of 2 M aqueous HCl with a precipitate being seen. The mixture was then taken up in water (200 ml) and freeze dried for 24 h to reveal an orange fluffy solid which was purified by flash column chromatography in the solvent system EtOAc followed by 10 EtOAc : 1 MeOH to afford (69) as an orange-yellow solid (0.28 g, 50%); m.p. 197°C; TLC (silica gel 10 EtOAc : 1 MeOH) Rf = 0.55; I.R. υ

max 3480 cm^{-1}, 3140 cm^{-1}, 1710 cm^{-1}, 1660 cm^{-1}, 1640 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 1.37 (2 H, m, CH_2), 1.62 (2 H, q, J 6.6Hz, purine-CH_2), 1.76 (2 H, q, CH_2), 2.73 (2 H, t, J 7.7 Hz, CH_2-NH), 3.24 (3 H, s, N-CH_3), 3.40 (3 H, s, N-CH_3), 3.53 (2 H, m, CH_2-NH), 6.56 (2 H, dd, 2' and 7'-H_2), 6.61 (2 H, d, xanthene 1', 8'-H_2), 6.70 (2 H, d, J 5.1 Hz, xanthene 4' and 5'-H_2), 7.20 (1 H, d, J 8.2 Hz, Ar-3H), 7.73 (1 H, m, Ar-4H), 8.12 (1 H, br, N-H), 8.25 (1 H, br, Ar-6H), 9.93 (1 H, br, CH_2-NH), 10.14 (2 H, s, 2 x OH), 13.21 (1 H, br, purine-NH); m/z 655 (M^+). Anal Calcd for ($C_{33}H_{30}N_6O_7S$): C, 60.53; H, 4.62; N, 12.84. Found C, 60.61; H, 4.57; N, 12.72.

5-{N-[8-(2,4-Dimethyl-1,3dioxopurin-8-yl)octyl]thioureido}-3',6'-dihroxyspiro(iso-benzofuran-1[3H],9'-[9H]xanthen)-3-one (70)

3'-6'-Dihydroxy-5-isothiocyanatospiro(isobenzofuran-1[3H],9'-[9H]xanthen-3-one (fluoresecein-5-isothiocyanate) (0.20 g, 0.52 mmol) was suspended in water (12 ml) and brought to pH 9.0 by dropwise addition of 1 M aqueous K_2CO_3. To this stirred solution, the 8-(8-Aminooctyl)-2,4-dimethylpurine-1,3-dione hydrobromide (0.2 g, 0.52 mmol) in 2:1 H_2O/dioxan (6 ml) was added over a 30 min period. The reaction was stirred at ambient temperature until the disappearence of starting amine with the pH being carefully at 9.0 by dropwise addition of 1 M aqueous K_2CO_3 throughout the course of the reaction.

After 3 h stirring at ambient temperature, the solution was acidified to pH 6.0 by dropwise addition of 2 M aqueous HCl with a precipitate being seen. The mixture was taken up in water (200 ml) and freeze dried for 24 h to reveal an orange fluffy solid which was purified by flash column chromatography in the solvent system EtOAc followed by 10 EtOAc : 1 MeOH to afford (70) (0.26 g, 72%)as an orange yellow solid. m.p. 182°C, TLC (silica gel 5 EtOAc : 1 MeOH) Rf = 0.65, I.R.υmax 3500-3100 cm^{-1}, 2960 cm^{-1}, 2880 cm^{-1}, 1710 cm^{-1}, 1660 cm^{-1}, 1610 cm^{-1}; ^1H NMR δ

[$(CD_3)SO$] 1.30 (8 H, br, 4 × CH_2), 1.55 (2 H, m, $NHCH_2CH_2$), 1.66 (2 H, m, purine-CH_2CH_2), 2.67 (2 H, t, J 7.3 Hz, purine-CH_2), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 3.49 (2 H, m, CH_2-NH), 6.53 (2 H, dd, 2' and 7'-H_2), 6.58 (2 H, d, xanthene 1', 8'-H_2), 6.68 (2 H, d, J 2.2 Hz, xanthene 4', 5'-H_2), 7.19 (1 H, d, J 8.4 Hz, Ar-3H), 7.70 (1 H, m, Ar-4H), 8.27 (1 H, br, Ar-6H), 10.13 (2 H, s, 2 x OH); m/z 696.8 (M^+). Anal Calcd for ($C_{36}H_{36}N_6O_7S$): C, 62.05; H, 5.21; N, 12.01. Found C, 62.11; H, 5.27; N, 12.02.

5-(N-(9-(2,4-Dimethyl-1,3-dioxopurin-8-yl)nonylthioureido)-3',6'-dihydroxyspiro(iso-benzofuran-1[3H],9'-[9H]xanthen)-3-one (71)

3'-6'-Dihydroxy-5-isothiocyanatospiro(isobenzofuran-1[3H],9'-[9H]xanthen-3-one (fluoresecein-5-isothiocyanate) (0.14 g, 37 μmol) was suspended in water (10 ml) and brought to pH 9.0 by dropwise addition of 1 M aqueous K_2CO_3. To this stirred solution, the 8-(9-Aminononyl)-2,4-dimethylpurine-1,3-dione hydrobromide (0.15 g, 0.37 mmol) in 2:1 H_2O/dioxan (5 ml) was added over a 30 min period. The reaction was stirred at ambient temperature until the disappearence of starting amine with the pH being carefully maintained at 9.0 by dropwise addition of 1 M aqueous K_2CO_3 throughout the course of the reaction.

After 3 h stirring at ambient temperature, the reaction was complete. The solution was acidified to pH 6.0 by dropwise addition of 2 M aqueous HCl. Water (30 ml) was added and the mixture was freeze-dried for 24 h to reveal an orange fluffy solid (0.47 g) which was purified by flash column chromatography in the system EtOAc followed by 10 EtOAc : 1 MeOH to afford (71) (0.18 g, 68%) as an orange yellow solid. m.p.178°C; I.R υmax 3400-3000 cm^{-1}, 2940cm^{-1}, 2860 cm^{-1}, 1740 cm^{-1}, 1700 cm^{-1}, 1650 cm^{-1}, 1610 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$)] 1.29 (10 H, br, 5 × CH_2), 1.56 (2 H, m, $NHCH_2CH_2$), 1.68 (2 H, m, purine-CH_2CH_2), 2.67 (2 H, t, J 7.5 Hz,

purine-CH_2), 3.22 (3 H, s, N-CH_3), 3.41 (3 H, s, N-CH_3), 3.48 (3 H, s, N-CH_3), 3.48 (2 H, m, $NHCH_2$), 6.56 (2 H, dd, J 8.8 and 2.2 Hz, xanthene 2', 7'-H_2), 6.59 (2 H, d, J 8.8 Hz, xanthene 1', 8'-NH_2), 6.67 (2 H, d, J 2.2, xanthene 4', 5'-H_2), 7.17 (1 H, d, J 8.4 Hz, Ar-3H), 7.70 (1 H, m, Ar-4H), 8.06 (1H, br, NH), 8.22 (1 H, brs, Ar-6H), 9.86 (1 H, br, CH_2NH), 10.13 (2 H, s, 2 × OH), and 13.15 (1 H, purine NH); m/z 710.3 (M^+); Anal cald for ($C_{37}H_{38}N_6O_7S$): C, 62.52; H, 5.39; N, 11.82. Found C, 65.45; H, 5.41; N, 11.75.

5-(N-{2-[2-(2,4-dimethyl-1,3-dioxopurin-8-ylmethoxy)ethoxy]ethyl}thioureido)-3',6'-dihydroxyspiro(isobenzofuran-1[3H],9'-[9H]xanthen)-3-one (72)

3'-6'-Dihydroxy-5-isothiocyanatospiro(isobenzofuran-1[3H],9'-[9H]xanthen-3-one (fluoresecein-5-isothiocyanate) (0.21 g, 0.53 mmol) was suspended in water (12 ml) and brought to pH 9.0 by dropwise addition of 1 M aqueous K_2CO_3. To this stirred solution 8-{2-[2-(2-Aminoethoxy)ethoxy]ethyl}-2,4-dimethylpurine-1,3-dione hydrobromide (0.2 g, 0.53 mmol) in 2:1 H_2O/dioxan (6 ml) was added over a 30 min period. The reaction was stirred at ambient temperature until the disappearence of starting amine with the pH being carefully maintained at 9.0 by dropwise addition of 1 M aqueous K_2CO_3 throughout the course of the reaction.

After 3 h stirring at ambient temperature the reaction was complete, the solution was acidified to pH 5.0 by dropwise addition of 2 M aqueous HCl. The mixture was taken up in water (200 ml), freeze-dried for 24 h to reveal an orange fluffy solid which was purified by flash column chromatography in the solvent system EtOAc followed by 10 EtOAc : 1 MeOH to afford (56) (0.28 g, 77%) an orange yellow solid. m.p. 197°C; TLC (silica gel 10 EtOAc : 1 MeOH) Rf = 0.58; I.R. υmax 3400-3200 cm^{-1}, 1740 cm^{-1}, 1700 cm^{-1}, 1650 cm^{-1}, 1610 cm^{-1}; ^1H NMR δ [$(CD_3)_2SO$] 3.22 (3 H, s, NCH_3), 3.41 (3 H, s, N-CH_3), 3.63 (8 H, m, $OCH_2CH_2OCH_2CH_2$), 4.54 (2 H, s,

purine-CH_2), 6.56 (2 H, dd, J 8.8 and 2.2 Hz, xanthene 2', 7'-H_2), 6.59 (2 H, d, J 8.8 Hz, xanthene 1', 8'-H_2), 6.66 (2H, d, J 2.2 Hz, xanthene 4', 5'-H_2), 7.18 (1 H, d, J 8.4 Hz, Ar-3H), 7.73 (1 H, brd, J 8.5 Hz, Ar-4H), 8.1 (1 H, br, CH_2NH), 8.26 (1 H, br, Ar-6H), 10.05 (1 H, br, NH), 10.14 (2 H, s, 2 × OH), 13.6 (1 H, purine NH); m/z 687 (M^+). Anal calcd for $C_{33}H_{30}N_6O_9S$: C, 57.71; H, 4.41; N, 12.24. Found C, 57.61; H, 4.45; N, 12.22.

2,2-Dimethyl-8-{5-[5-(dimethylamino)naphthalene-1-sulphonylamino]pentyl}purine-1,3-dione (73)

To a solution of 8-(5-Aminopentyl)-2,4-dimethylpurine-1,3-dione hydrobromide (0.2 g, 0.58 mmol) in 2:1 H_2O/dioxan (12 ml), 0.5 M aqueous Na_2CO_3 was added dropwise until pH was maintained at 9.5. To this mixture dansyl chloride (0.16 g, 0.58 mmol) in dioxane (10 ml) was added and the solution stirred at ambient temperature within the pH range 9.5-10.0 until the disappearence of the starting hydrobromide.

After 3 h stirring at ambient temperature, the pH was adjusted to 7.0 by the dropwise addition of 2 M aqueous HCl, water (25 ml) was added and the resulting solution freeze-dried for 24 h. To this solid ethyl acetate was added, the solution washed once with water, dried and the solvent evaporated to reveal (73) (0.16 g, 55%) as green-yellow solid. m.p. 183°C; TLC (silica gel 10 EtOAc : 1 MeOH) Rf = 0.55; ^{1}H NMR δ [$(CD_3)_2SO$] 1.14 (2 H, m, $CH_2CH_2C\mathit{H}_2CH_2CH_2$), 1.29 (2 H, quintet, J 7 Hz, $CH_2C\mathit{H}_2CH_2CH_2CH_2$), 1.46 (2 H, quintet, J 7 Hz, $CH_2CH_2CH_2C\mathit{H}_2CH_2$), 2.76 (2 H, q, J 5.9 Hz, $NHCH_2$), 2.81 (6 H, s, $N(CH_3)_2$), 3.22 (3 H, s, purine N-CH_3), 3.39 (5 H, br, purine N-CH_3 + purine-CH_2), 7.24 (1 H, d, J 7.3 Hz, Ar-H), 7.58 (1 H, t, 8.1 Hz, Ar-H), 7.61 (1 H, t, J 7.7 Hz, Ar-H), 7.87 (1 H, t, J 5.9 Hz, N*H*CH_2), 8.08 (1 H, d, J 7.3 Hz, Ar-H), 8.29 (1 H, J 8.4 Hz, Ar-H), 8.44 (1 H, d, J 8.5 Hz, Ar-H), 13.1 (1 H, br, purine-NH).

5-{6-Chloro-2-[5-(2,4-dimethyl-1,3-dioxopurin-8-yl)pentylamino]-1,3,5-triazin-4-yl-amino}-3',6'-dihydroxyspiro(isobenzofuran-1-[3H],9'-[9H]xanthen-3-one (74)

5-{4,6-Dichloro-1,3,5-triazin-4-ylamino}-3',6'-dihydrospiro(isobenzofuran-1[3H],9'-[9H]xanthen-3-one (0.31 g, 0.58 mmol) in water (12 ml) was brought to pH 8.0 by dropwise addition of 1 M aqueous K_2CO_3. To this mixture a solution of the 8-(5-Aminopentyl)-2,4-dimethylpurine-1,3-dione hydrobromide (0.20 g, 0.58 mmol) in 2:1 H_2O/dioxan (6 ml) was added over a 10 min period and the mixture stirred at ambient temperature for 5h with the pH being controlled at pH 8.0 by further addition of 1 M aqueous K_2CO_3.

After completion of the reaction the pH was reduced to 5.0 by the dropwise addition of 2 M aqueous HCl, water was added and the resulting aqueous solution freeze-dried for 24 h. The resulting solid was then purified by flash column chromatography in EtOAc then flushed with 10 EtOAc: 1 MeOH, collected, with evaporation of the solvent affording () (0.18 g, 68%) as a yellow solid. m.p. 210°C; TLC (silica gel 10 EtOAc : 1 MeOH) Rf = 0.43; ^{1}H NMR δ [$(CD_3)_2SO$] 1.29 (2 H, m, $CH_2CH_2C\mathit{H}_2CH_2CH_2$), 1.47 (2 H, m, NCH_2CH_2), 2.54 (2 H, m, $NHCH_2$), 3.10 (2 H, m, CH_2), 3.25 (3 H, s, purine N-CH_3), 3.29 (5 H, br, purine N-CH_3 + purine-CH_2), 6.41 (2 H, dd, xanthene 2' and 7'-H_2), 6.48 (2 H, d, xanthene 1' and 8'-H_2), 6.48 (2 H, d, xanthene 4' and 5'-H_2), 7.14 (1 H, d, J 8.4 Hz, Ar-3H), 10.02 (2 H, s, 2 × OH).

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